BIOLOGICAL MARKERS DEMONSTRATE UTILITY AND PREDICTIVE VALUE IN INFLAMMATORY BOWEL DISEASE

A Thesis Submitted to the College of

Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

In the College of Pharmacy and Nutrition

University of Saskatchewan

Saskatoon

By

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ABSTRACT

Biological markers ("biomarkers") may have applications in inflammatory bowel disease (IBD), a chronic disease of the gastrointestinal tract. Clinicians are presented with several challenges when treating IBD. Instead of performing expensive and invasive endoscopic procedures - if even possible, as resources for these procedures can be limited - biomarkers could be used to diagnose, assess disease activity and prognosis, and guide medical therapy, particularly in situations where novel biologics are involved. At this time, the use of biomarkers is limited, since few have been useful in predicting disease severity, prognosis and therapeutic response in IBD. Previous research cohorts studying biomarkers are limited due to varying heterogeneity between subjects that confounds the results since patients have variable disease courses.

The main aim of this work was to evaluate the utility of biomarkers in IBD. To do this, biomarkers were included into a composite score with other patient reported outcomes (PRO) to predict endoscopic disease activity. Next, we examined the role of biomarkers in newly diagnosed IBD. Lastly, fecal calprotectin (FC) was evaluated in healthy pregnant and IBD patients, establishing reference values and practicality in this clinical group. We also studied the relationship between biomarkers and environmental factors, such as fecal microbiota. We hypothesized biomarker concentration would be elevated with increased clinical and endoscopic measures, and predictive of response to medical therapy in newly diagnosed patients. Additionally, we theorized the inclusion of biomarkers into composite scores would outperform existing scoring models in predicting endoscopic severity. Furthermore, FC levels would be below the limit of detection in healthy pregnancy and elevated in IBD pregnancy.

The inclusion of biomarkers into composite scoring models outperformed existing clinical scores. In newly diagnosed patients, modest relationships were found between biomarkers and clinical and endoscopic markers of disease. Lastly, the presence of FC was elevated in pregnant IBD and not significant in healthy pregnancy; thus, FC is useful in IBD and pregnancy. Our work confirmed the significance of biomarkers in several clinical areas of IBD, along with the issues presented in recruiting newly diagnosed patients in small research centres. Future work will incorporate biomarkers into medical triage and as an endpoint in nutritional interventions.

ACKNOWLEDGEMENTS

I would first like to acknowledge the role my supervisors, Dr. Gordon Zello and Dr. Jennifer Jones, played in my doctoral research and development as a professional. Dr. Zello took a chance on me after reading a "well written e-mail" I crafted and always fought for me throughout my entire academic experience. Dr. Jones was very patient and kind in providing feedback to a rookie in the complex research area of IBD. I will have fond memories of both of my supervisors and the impact they had on me.

My advisory committee members played a vital role in not only guiding me through graduate school, but helping shape my ever changing PhD project. First of all, Dr. Alcorn consistently went above and beyond her role as a committee member, stepping in whenever feedback was required. Additionally, I'd like to thank the other members of my committee, Dr. W. Dahl, Dr. C. Rodgers, Dr. S. Fowler, and chairs Dr. R. Dobson and Dr. S. Whiting. Strangely, I looked forward to committee meetings – discussions about my work were motivating and helpful.

I would like to thank the faculty and staff of the College of Pharmacy and Nutrition (CoPN). From day one, I felt welcomed and rarely did I ever have a question or concern go unanswered - particularly, Mrs. M. Beazley, Ms. J. Huck, Dr. F. Remillard, and Dr. E, Smith-Windsor took care of my administrative questions. From a technical standpoint, I wouldn't have been able to finish without the contributions from Deb Michel. She truly is an indispensable member of our College. Juan-Nicolas Sanchez, Sam Stewart and Pat Paulo, from the College of Medicine were also tremendously helpful at various points of my project. My gratitude is further extended to my colleagues from the CoPN who provided assistance and support throughout this experience; Ms. S. Mitchell, Mrs. T. Phillips, Dr. E. Ling, Mrs. J. Wright, Mr. C. Arnold, and Mr. A. Alemmari.

I would like to thank the numerous funds who supported my education, particularly the Rhea Ritchie Scholarship and Teacher Doctoral Fellowship through the CoPN and College of Graduate Studies and Research (CGSR), the College of Medicine and the Natural Sciences and Engineering Research Council of Canada (NSERC).

Finally, I would like to thank my parents, Janet and Richard, sister, Makenzie, and extended family and friends for their support throughout this journey. Lastly, none of this would have been possible without my beautiful fiancée, Amy, pushing and guiding me to be better. I truly appreciate all of these contributions, without them I wouldn’t have finished this pursuit.

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AMDR	acceptable macronutrient distribution range
AUC	area under the curve
FC	fecal calprotectin
CD	Crohn's disease
CDEIS	Crohn's disease index of severity
CDAI	Crohn's disease Activity Index
CRF	case report form
CRP	C - reactive protein
e	base of the natural logarithm
EWCFG	Eating Well with Canada's Food Guide
FODMAPS	Fermentable Oligo-, Di- and Monosaccharides and Polyols
FDA	Food and Drug Administration
FFQ	food frequency questionnaire
FMT	fecal microbiota transplant
GI	glycemic index
GC	gas chromatography
GL	glycemic load
HBI	Harvey Bradshaw Index
HP	healthy pregnant
HRQL	health related quality of life
hsCRP	high-sensitivity C-reactive protein
IBD	Inflammatory Bowel Disease
IBS	irritable bowel syndrome
IL	interleukin
Lf	lactoferrin

MDIBDC	Multidisciplinary Inflammatory Bowel Disease Clinic
na	not applicable
nd	no detection
NPV	negative predictive value
POC	point-of-care
PPV	positive predictive value
PRO	patient reported outcome
PT	Powell-Tuck index
RDA	recommended daily allowance
ROC	receiver operating characteristic
QL	quantification limit
QOL	quality of life
ROS	reactive oxygen species
RUH	Royal University Hospital
SCFA	short-chain fatty acid
SES-CD	simple endoscopic score for Crohn’s disease
TNF	tumour necrosis factor
UC	ulcerative colitis

CHAPTER 1

INTRODUCTION

1.1 Rationale

Inflammatory Bowel Disease (IBD) is a major health concern in developed countries, including Canada. IBD encompasses a multisystem group of disorders, with underlying chronic inflammation and specific clinical and pathological features, that primarily affects the gastrointestinal tract (Burri & Beglinger, 2012). Classical IBD consists of two main forms: Crohn's disease (CD) and ulcerative colitis (UC). Although grouped together, CD and UC differ with respect to some important clinical, immunophenotypic and pathologic characteristics, including disease location, complications and histology (Bamias et al., 2005; Podolsky, 2002).

An aberrant immune response, a product of environmental and genetic factors, is central to pathogenesis and etiology of IBD (de Souza and Fiocchi, 2015). This inappropriate and ongoing response is driven by the presence of normal luminal bacteria, and is propagated by abnormalities in both the mucosal barrier function and immune system (Podolsky, 2002). Environmental influences occur at the local microenvironment (enteric microflora) and nutritional environment (Shanahan, 2001).

Chronic inflammation in the gastrointestinal tract is associated with diarrhea, abdominal pain and variable amounts of bleeding, in addition to generic systemic inflammatory manifestations (i.e. fever, weight loss), as well as extraintestinal manifestations (i.e. arthritis, uveitis) (Roy, 1997; de Mattos et al., 2015). Therefore, clinical assessment of inflammation is key to the diagnosis and monitoring of IBD (Konikoff & Denson, 2006). Since inflammation of the gastrointestinal tract may be subclinical, and thus difficult to observe and assess by patients or physicians, various techniques and systems have been developed to quantify the severity and extent of this inflammation (Konikoff & Denson, 2006). Medical therapy in IBD focuses on halting the acute inflammatory response by interfering with the immune response that creates this inflammation. Clinicians apply a combination of symptom scoring, clinical examination, laboratory indices, radiology, in addition to endoscopy with biopsy to make a diagnosis, assess severity and predict the outcome of disease (Vermeire, Van Assche, & Rutgeerts, 2006).

Although expensive and invasive, endoscopy is the gold standard for measuring inflammatory disease activity in IBD. However, due to cost, access to resources and patient burden, clinicians rely on the assessment of clinical symptoms. A patient-reported outcome (PRO) is any report or score supplied solely by the patient about a health condition or treatment (Feagan, Hanauer, Coteur, & Schreiber, 2011; Guyatt et al., 1989). Although assessment of clinical symptoms is important for the management of IBD, patient-derived and clinical scores of disease activity are subjective. Additionally, they are ineffective at discriminating between other medical conditions, like Irritable Bowel Syndrome (IBS) and active inflammatory disease (Lahiff et al., 2013).

The clinical symptoms of IBD, particularly CD, are not specific and unfortunately no hallmark sign or symptom exists (Burri & Beglinger, 2012). There is considerable overlap in the symptoms found in functional disorders, such as IBS, and organic diseases like IBD. Thus, differentiating between organic and functional disease is difficult and often requires a colonoscopy with histology. Laboratory markers that are specific to IBD and could accurately detect inflammation and monitor disease would be useful clinically (Abraham & Kane, 2012). A marker, or set or markers, to fulfill this role would provide an objective measure of disease activity while avoiding invasive and expensive procedures (Vermeire et al., 2006). Currently, few biomarkers are clinically useful in terms of their predictive power for disease severity, prognosis and therapeutic response in IBD (Jones & Loftus, 2007; Sands et al., 2003). Additionally, previous biomarker studies in IBD have excluded pregnant patients.

Previous cohort studies in IBD exploring predictors of disease course have had limitations due to the heterogeneity and confounding effect of numerous subject and disease-related characteristic of the subjects within the sample population. When studying a population of patients with long-established disease, they are all subject to different medications and dietary intakes, complications and surgeries, all of which distort the usefulness and calculation of biomarker concentrations and measures of disease activity. Thus, it is important to study newly diagnosed patients – a more homogenous sample population limits some of the confounding found in prevalent cohorts.

1.2 Hypotheses

This thesis is divided into four main sections, which tested the following hypotheses (hypothesis is numbered for organizational purposes):

BIOMARKER BASED MODELS (Ch. 4)

- Biomarkers have a strong, positive correlation with endoscopic disease activity **(4-1)**
- Biomarker-based models are more accurate than the Crohn's disease Activity Index (CDAI) for predicting endoscopic disease activity **(4-2)**

INCEPTION COHORT (Ch. 5)

- Fecal and serological biomarker concentrations are elevated with active clinical and endoscopic disease activity **(5-1)**
- Fecal and serological biomarker concentrations are predictive of disease activity at repeated measurements **(5-2)**
- Food groups and nutrient intake are lower in newly diagnosed population IBD patients compared with reference standards **(5-3)**
- Glycemic index and glycemic load have a strong, positive correlation with fecal biomarkers in newly diagnosed IBD patients **(5-4)**

CALPROTECTIN IN PREGNANCY (Ch. 6)

- Fecal calprotectin is not detectable in healthy pregnant women **(6-1)**
- Fecal calprotectin is a feasible and useful marker of intestinal inflammation in pregnant IBD patients **(6-2)**

FECAL MICROBIOTA AND SHORT CHAIN FATTY ACIDS (SCFA) IN IBD (Ch. 7)

- Fecal SCFA are reduced with increased clinical and endoscopic disease activity **(7-1)**

1.3 Objectives

This thesis is divided into four main sections, with the following objectives:

BIOMARKER BASED MODELS (Ch. 4)

- Analyze individual components of the CDAI (both PRO- and lab-based), biomarkers and other variables of interest in predicting endoscopic disease activity
- Create a new composite scoring formula for the SES-CD including biomarkers

INCEPTION COHORT (Ch. 5)

- Assess feasibility of recruiting newly diagnosed cohorts in small research centres
- Provide a nutritional assessment, observing food group and nutrient intake, and carbohydrate quality, in newly diagnosed, Canadian IBD patients

CALPROTECTIN IN PREGNANCY (Ch. 6)

- To determine the median concentrations, per trimester, of FC in normal healthy pregnancies.
- To assess the utility of FC for following disease activity in pregnant patients with IBD
- Assess if FC concentration correlates with clinical outcomes

FECAL MICROBIOTA AND SCFA IN IBD (Ch. 7)

- Analyze the impact disease activity has on fecal organic acids and fecal microbiota in newly diagnosed IBD patients
- Examine the relationship between *Faecalibacterium prausnitzii* and fecal butyric acid is in newly diagnosed patients

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

IBD is a major health concern in developed countries, including Canada. The objective of this literature review is to introduce the clinical research area of IBD, with a specific emphasis on newly emerging research surrounding inflammatory biomarkers, and how existing research strategies may be tailored to investigate long-standing questions associated with IBD.

2.1.1 Inflammatory Bowel Disease: IBD encompasses a multisystem group of disorders, with underlying chronic inflammation and specific clinical and pathological features, that primarily affects the gastrointestinal tract (Burri & Beglinger, 2012). An aberrant immune response, a product of environmental and genetic factors, is central to pathogenesis and etiology of IBD (de Souza and Fiocchi, 2015) . This inappropriate and ongoing response is driven by bacteria and is propagated by abnormalities in both the mucosal barrier function and immune system (Podolsky, 2002).

Chronic inflammation leads to IBD related symptoms. Therefore, clinical assessment of inflammation is key to the diagnosis and monitoring of IBD (Konikoff & Denson, 2006). Periodic bouts of diarrhea, abdominal pain and variable amounts of bleeding are associated with chronic inflammatory conditions of the gastrointestinal tract, in addition to generic systemic inflammatory manifestations (i.e. fever, fatigue and weight loss) as well as extraintestinal manifestations (i.e. arthritis, uveitis) (Roy, 1997; de Mattos et al., 2015). Since inflammation may be subclinical, and thus difficult to observe and assess by patients or physicians, various techniques and systems have been developed to quantify the severity and extent of this inflammation (Konikoff & Denson, 2006). Clinicians apply a combination of symptom scoring,

clinical examination, laboratory indices, radiology, in addition to the current gold- standard of endoscopy with biopsy to make a diagnosis, assess severity and predict the outcome of disease (Vermeire et al., 2006).

Left untreated, IBD can cause many debilitating symptoms and complications leading to significant disease-related morbidity and impaired quality of life (Munkhom & Binder, 2004). The prevalence of IBD in Canada has been estimated to be amongst the highest in the world, with increased mortality in patients with CD (Bernstein et al., 2006). Bernstein and colleagues (2006) estimated that 13 and 12 cases of CD and UC, respectively, are diagnosed per 100,000 Canadians every year. Due to the early age of diagnosis for this lifelong disease, IBD is a large financial burden due to its effect on health-related quality of life (HRQL), work productivity and direct medical costs (Gregor et al., 1997).

Classical IBD consists of two main forms: CD and UC. Although generally grouped together, CD and UC differ with respect to some important clinical, immunophenotypic and pathologic characteristics, including disease location, complications and select histopathological features (Bamias et al., 2005; Podolsky, 2002) (**Table 2.1**).

Table 2.1 Key and distinguishing factors of Crohn's disease and ulcerative colitis

Feature	Ulcerative colitis	Crohn's disease
Clinical Features		
Fever	Fairly common	Common
Abdominal pain	Varies	Common
Diarrhea	Very common	Fairly common
Rectal bleeding	Very common	Fairly common
Weight loss	Fairly common	Common
Malnutrition	Fairly common	Common
Perianal disease	Absent	Fairly common
Abdominal mass	Absent	Common
Growth failure	Occasional	Common
Site		
Colon	Exclusively	2/3 of patients
Ileum	Infrequent (backwash ileitis)	2/3 of patients
Jejunum	Never	Infrequent
Stomach or	Never	Infrequent
Esophagus	Never	Infrequent
Intestinal		
Stricture	Unknown	Common
Fistula	Absent	Fairly common
Toxic mega colon	Infrequent	Uncommon
Perforation	Unknown	Uncommon
Cancer	Common	Fairly common
Endoscopic findings		
Friability	Very common	Fairly common
Aphthous and linear	Absent	Common
Cobblestone	Absent	Common
Pseudopolyps	Common	Fairly common
Rectal involvement	Very common	Fairly common
Radiologic findings		
Distribution	Continuous	Discontinuous, segmented
Ulceration	Fine, superficial	Deep, submucosal
Fissures	Absent	Common
Strictures or fistulas	Rare	Common
Ileal involvement	Dilated	Narrow, nodular

Adapted from Podolsky (2002)

2.1.2 Crohn's disease: CD is a chronic inflammatory condition characterized by a transmural, segmental or patchy inflammatory process that can affect any part of the gastrointestinal tract (Van Assche et al., 2010). CD may be defined by location (terminal ileal, colonic, ileocolonic, upper gastrointestinal) or by pattern of disease (inflammatory, stricturing, or fistulising) (Van Assche et al., 2010). Additionally, CD is distinguished by macrophages that typically form non-caseating granulomas. Inflammation can occur anywhere in gastrointestinal tract; however, the terminal ileum is most commonly involved in CD, with the earliest mucosal lesions appearing over Peyer's patches (Xavier & Podolsky, 2007).

2.1.3 Ulcerative colitis: UC is a chronic inflammatory condition causing continuous mucosal inflammation of the colon without granulomas on biopsy, affecting the rectum and extending proximally to a variable extent of the colon (Silverberg et al., 2005; Stange et al., 2008). Three major phenotypes of UC have been described: proctitis (limited to the rectum), left-sided disease (distal to the splenic flexure), and pancolitis (extending past the splenic flexure) (Podolsky, 2002). Regardless of the extent of disease, the appearance of blood in stool is a notable characteristic of UC (Roy, 1997). In addition to the absence of granulomas, histology reveals the presence of a significant number of neutrophils within the lamina propria and the crypts of the gastrointestinal mucosa, where they form micro-abscesses. UC is characterized by considerable superficial mucosal ulceration, as opposed to the transmural nature of CD (Xavier & Podolsky, 2007).

2.1.4 Current Medical Model and Treatment of Disease: In response to an acute inflammatory stimulus, the body will mount an acute phase reaction by the up or down regulation of various acute phase proteins. Upon resolution of this event, the acute phase proteins will return to baseline levels, albeit not all at the same speed (Gabay & Kushner, 1999). More specifically, the presence of active gut inflammation in IBD is associated with an acute phase reaction and migration of leucocytes to the gut. This event translates into production of several proteins, some that are directly involved in the immune response and others that are by-products, detected in serum or stools of IBD patients (Mazlam & Hodgson, 1992; Niederau, Backmerhoff, Schumacher, & Niederau, 1997; Pepys, Druget, & Klass, 1977; J. Tibble et al., 2000). Medical therapy in IBD aims to stop the acute inflammatory response, usually through drug therapy, by interfering with the immune response that creates this inflammation. However, this approach is limited and includes the use of nonspecific anti-inflammatory medications, such as 5-ASAs (e.g. mesalazine), and immunosuppressive medications (e.g. azathioprine) (Bamias et al., 2005; Roberfroid et al., 2010). Novel biologic therapeutics, such as infliximab, an antibody that blocks tumour necrosis factor (anti-TNF agent), have been used effectively in the treatment of patients greatly improving HRQL (Bamias et al., 2005; Feagan, Yan, Bala, Bao, & Lichtenstein, 2003; Hanauer et al., 2002; Sandborn et al., 2004; Sands et al., 2004). Recently, new therapy has been introduced due to the discovery of new targets, such as anti-IL12-23 agents (e.g. Ustekinumab) and anti-integrins (e.g. Vedolizumab) (Feagan et al., 2013; Sandborn et al., 2012). However, the effectiveness of these agents is accompanied by high cost and the possibility of serious adverse events. Additionally, natural history studies have shown that approximately half of IBD patients will follow a mild disease course (Farmer, Whelan, & Fazio, 1985; Munkholm, 1997). Despite this knowledge, some experts are still opting for a more wide

spread application of early and aggressive use of biologics ("top-down therapy") (D'Haens et al., 2008). Some experts advocate for this approach since treating some patients earlier will give a better response to medication, thus better disease control, attenuated progression and fewer complications (D'Haens, Sartor, Silverberg, Petersson, & Rutgeerts, 2014). However, this approach may unnecessarily expose half the IBD population to a biologic or immunosuppressive medication. The ability to identify patients with active inflammation, and furthermore, stratify patients that are likely to have a severe disease course, would help a physician select appropriate medical therapies. This type of clinical research may help minimize health care cost and adverse outcomes to these therapeutic agents.

2.1.5 Populations with IBD: Population based studies have shown IBD to be a disease of the young (Bernstein et al., 2006). Bernstein et al. (2006) observed peak incidence of CD between 20-29 years, and although there was no peak age for prevalence or incidence for UC, rates rise around 30 years of age. Consequently, management of IBD during pregnancy is very common. Endoscopy can be safely performed during pregnancy; however, most patients and clinicians prefer to avoid invasive measures during this time (Fowler, Jones, Martel, & Mytopher, 2013). Poorly controlled IBD during pregnancy is associated with poor pregnancy outcomes including preterm delivery and small for gestational aged babies, but pregnancy outcomes when IBD is well controlled are good. Therefore, non-invasive surrogate markers for intestinal inflammation would be vital to the care of pregnant IBD patients.

2.2 Clinical Research in IBD

2.2.1 Measures of Disease Activity and Study Outcomes: Currently, several aspects of the management of IBD present challenges to gastroenterologists and health care teams. A combination of colonoscopy with biopsies (for histology), symptom-based scoring, clinical examination and laboratory indices or markers, are used to make a diagnosis and subsequently assess the severity, predict the outcome and select appropriate medications in the treatment of the disease (Vermeire et al., 2006).

2.2.1.1 Colonoscopy

The current "gold" standard for assessing and detecting intestinal inflammation is performing an endoscopy. This technique allows a gastroenterologist to visually examine the gastrointestinal tract. During this procedure, biopsy samples from the mucosa can be obtained for histology. In addition to giving a visual assessment of the affected areas, colonoscopy provides information on the location, extent and severity of the disease. However, colonoscopy is an invasive procedure, one that increases the risk of perforation and bleeding, and requires a health-care team to perform, in addition to a high patient burden and substantial health-care cost. These limitations prevent frequent assessment of disease activity by colonoscopy (Konikoff & Denson, 2006).

IBD is characterized by the presence of extensive ulceration in the gut. Until recently, the focus of medical treatment in IBD was on improving the signs and symptoms of the disease, instead of treating the ulceration of the bowel (Rutgeerts, Vermeire, & Van Assche, 2007). Many of the complications encountered in IBD (i.e. perforation, bleeding, and fistulas) are a result of this ulceration. Therefore, a logical objective of medical treatment would aim for mucosal healing in parallel with clinical remission (Rutgeerts et al., 2007). A mounting body of evidence

suggests that mucosal healing, evaluated through colonoscopy, is a surrogate marker of sustained controlled CD (D'Haens et al., 2008; Rutgeerts et al., 2004; Van Assche et al., 2010). Therefore, the use of frequent endoscopic monitoring in the effort to assess mucosal healing is advocated (Van Assche et al., 2010).

Endoscopy, although considered the gold-standard and the most objective test available, is still a subjective measure that has been historically limited by interobserver variation (de Dombal & Softley, 1987). Some of the earliest versions of endoscopic activity scoring were not validated for use in clinical trials (Gomes, du Boulay, Smith, & Holdstock, 1986; Olaison, Sjödahl, & Tagesson, 1990). The Crohn's disease Endoscopic Index of Severity (CDEIS) is a validated scoring tool used for assessment in CD (Mary & Modigliani, 1989). Recently, a more simplified score, called the Simple Endoscopic Score for Crohn's disease (SES-CD), has been developed and also validated (Daperno et al., 2004). Sandborn et al. (2002) have performed a review of endoscopic indices in CD, in addition to other indices of disease activity. This review found that the CDEIS score to be the standard for assessing endoscopic healing of CD. However, the authors noted that more prospective research is needed to validate the prognostic relevance of the CDEIS, and, more importantly, will be required before endoscopic disease activity is utilized as a primary endpoint. D'Haens et al. (2007) have performed an extensive review of endoscopic indices in UC, such as the Simple Clinical Colitis Activity Index and Mayo Score. This review recommended that an endoscopic endpoint, used as a composite score in a larger assessment or scored separately, should be incorporated into the primary study endpoint. Furthermore, the authors added that an "optimal" scoring tool in UC has yet to be developed. The substantial complexity, with scores that include partial endoscopic and partial clinical scoring, and sheer abundance of scoring methods are beyond the scope of this literature review. However, even

though there is no perfect marker or test of disease activity, endoscopic assessment remains the standard in assessment of disease activity.

2.2.1.2 Patient Reported Outcomes and Clinical Indices

Assessments of patient symptoms are important for the clinical management of inflammation. However, patient-derived reports and clinical scores of disease activity are subjective. Furthermore, they may be influenced by other non-inflammatory features of the disease, (i.e. intestinal strictures, bile salt malabsorption) or even non-disease related occurrences, such as emotional state (Konikoff & Denson, 2006).

Clinical Indices in Crohn's disease

In the 1970's, experts involved with the National Cooperative Crohn's disease study, void of an index to score the severity of disease in CD, gathered to create a method to assess the response or lack of response to a treatment regimen (Best, Becktel, Singleton, & Kern, 1976). The CDAI, derived through a multiple regression model of 8-variables, has since been widely used in clinical trials in CD and is still used today (Sandborn et al., 2002). Scores of less than 150 indicate remission, whereas greater than 450 indicate severe disease. When compared with more objective endpoints of disease activity, such as laboratory indices and endoscopy, the CDAI has been shown to correlate poorly (Gomes et al., 1986; Jones, Loftus, Panaccione, Chen, Peterson, McConnell, et al., 2008; Kiss et al., 2011; Thia et al., 2008). Also, the CDAI score has been shown to be poorly reproducible, with a weak response to change over time (de Dombal & Softley, 1987). Additionally, more recent work has shown the CDAI to be ineffective at discriminating between IBS and active CD (Lahiff et al., 2013). The Harvey-Bradshaw Index (HBI) is a strongly correlated ($r = 0.90$) simplification of the CDAI, designed to make data collection and computation easier (Harvey & Bradshaw, 1980).

Clinical Indices in UC

In 1955, Truelove and Witts reported the results of a trial for treating active UC; in that they described an instrument they used to measure disease activity. The Truelove and Witts Severity Index is composed of six variables: number of stools per day; blood in stools; temperature; pulse; hemoglobin; and erythrocyte sedimentation rate (ESR) (Truelove & Witts, 1955). At present, it is useful only to broadly classify patients, as it does not sufficiently measure changes in disease activity.

In 1978, the Powell-Tuck index (PT), also known as the St. Mark's Index, was described (Powell-Tuck, Bown, & Lennard-Jones, 1978). The PT includes 10 clinical variables: general health, abdominal pain, bowel frequency, stool consistency, bleeding, anorexia, nausea or vomiting, abdominal tenderness, extra intestinal complications (eye, mouth, joint, skin), and temperature. The score ranges from 0 to 20, with remission being defined as 0, and a 2-point decrease recognized as a clinical improvement. This scoring method has not been validated.

In 1993, Hanauer and colleagues reported the results of a placebo-controlled trial in active UC that utilized the Physician Global Assessment (PGA) (Hanauer et al., 1993). The PGA is a multicomponent measure of disease activity, which utilizes the physician's assessment of the patient. Scores range from 1 to 6, with a treatment success defined as 1 or 2 and treatment benefit as 1. This score and accompanying definitions have not been validated.

In 1987, Schroeder and colleagues (Schroeder, Tremaine, & Ilstrup, 1987) reported the results of a placebo-controlled trial utilizing a measurement called the Mayo Score or Disease Activity Index (DAI). The score consists of 4 items: stool frequency, rectal bleeding, findings of flexible proctosigmoidoscopy and PGA. Scores range from 0 to 12 points. Complete remission is defined as a score of zero, meaning normal stool frequency, no rectal bleeding, the patient is

generally well, and a PGA score of 0. A partial response has also been defined. The Mayo Score and accompanying definitions have not been validated.

An extensive overview of the clinical indices and efficacy endpoints in UC has been reviewed by D'Haens and colleagues (D'Haens et al., 2007). There are a variety of clinical disease activity scores, some of that contain endoscopic sub-score components that are likely to increase accuracy of the score. However, none of these scores are validated.

2.2.2 Biological Markers: The clinical symptoms of IBD, particularly CD, are not specific and unfortunately no hallmark sign or symptom exists (Burri & Beglinger, 2012). There is considerable overlap in the symptoms found in functional disorders, such as IBS and organic diseases like IBD. Thus, differentiating between the organic and functional disease is difficult and often requires a colonoscopy with histology. Laboratory markers that are specific to IBD and could accurately detect inflammation and monitor disease would be useful clinically (Abraham & Kane, 2012). A marker, or set or markers, to fulfill this role would provide an objective measure of disease activity while avoiding invasive and expensive procedures (Vermeire et al., 2006).

A biological marker ("biomarker") is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working Group., 2001). Currently, few biomarkers are clinically useful in terms of their predictive power for disease severity, prognosis and therapeutic response in IBD (Jones & Loftus, 2007; Sands et al., 2003). The majority of current biomarkers lack the specificity for gastrointestinal inflammation or do not have the extensive research required to be clinically useful. In the context of IBD, biomarkers may be used to assess disease activity, severity, and predict the course of the disease

with low expense, high reliability and comparative ease to other assessments (Desai, Faubion, & Sandborn, 2007). Further information on the qualities of an ideal biological marker can be found in **Table 2.2**.

Table 2.2 Performance and qualities of an ideal marker

Performance	Qualities
Simple	Disease specific: identify at risk individuals and distinguish IBD from non-IBD
Easy to perform	Objectively measure disease activity
Not/minimally invasive	Predict disease course
Cheap	Monitor effect of treatment
Rapid	Prognostic value: asses morbidity/mortality
Reproducible between labs and individuals	

Adapted from Vermeire (2006)V.

Recently, an influx of research has been exploring the role of biomarkers for their diagnostic ability, as non-invasive measures of disease activity and their accuracy in predicting disease progression and response to medication. In diagnostics, one of the simplest tests utilizes the results of investigation to classify patients into two groups, due to the presence or absence of a sign (Altman & Bland, 1994). Within this test, the measures of sensitivity and specificity can be measured. These two measures are closely related to the concepts of Type I and II errors ("Sensitivity and specificity," 2013). The positive predictive value (PPV) assesses the performance of the test, measures of the proportion of true positive test to combined true and false positive results. The negative predictive value (NPV) is the proportion of subjects with a negative test result who are correctly diagnosed. **Figure 2.1** displays the diagnostic role of biomarkers ("Sensitivity and specificity," 2013).

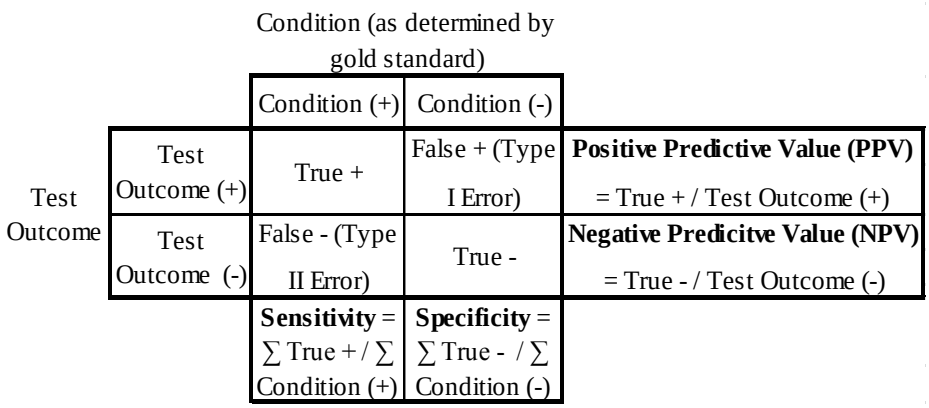

Figure 2.1 Diagnostics of biomarkers. Adapted from "Sensitivity and specificity" ("Sensitivity and specificity," 2013).

Serological markers have been shown to be problematic in terms of sensitivity and specificity. The transcriptional control of many serological markers, such as C-reactive protein, make them complex markers to assess and utilize. The various serological, fecal and novel biomarkers studied in clinical IBD research will now be discussed.

2.2.2.1 Serum Markers

As previously mentioned, the acute inflammatory response is marked by the upregulation of several plasma proteins (≈40 proteins) (Henriksen et al., 2008). A set of markers that could accurately assess inflammation and monitor disease activity would prove useful in IBD. In response to an acute phase stimulus, white blood cell count, platelet count, or erythrocyte sedimentation rate (ESR) are typically altered (Vermeire et al., 2006). Unfortunately, the drawbacks to these measurements are the lack of sensitivity and specificity for intestinal inflammation. Due to various physiological parameters, such as short half-life and high sensitivity, C-reactive protein (CRP) has been widely utilized and studied in IBD.

C-reactive protein

CRP, a pentameric protein consisting of five monomers, is one of the most vital acute phase proteins in humans (Vermeire, Van Assche, & Rutgeerts, 2004). CRP is produced almost exclusively by hepatocytes in the liver, although extra-hepatic production has been demonstrated (Henriksen et al., 2008). The main stimulus for CRP production is the pro-inflammatory cytokine interleukin-6 (IL-6), originating at the site of inflammation. This response is enhanced in combination with interleukin 1-beta (IL1-ß) and TNF-alpha (Henriksen et al., 2008; Vermeire et al., 2004). After binding to its ligand, the CRP–ligand complex activates the complement cascade and induces phagocytosis, fulfilling an important role in the innate human immune system. The full function of how CRP functions in disease is not well understood.

CRP is useful marker in the detection and follow-up of acute response events due to its short half-life (≈19 hours) (Vermeire et al., 2006). Thus, CRP is suitable to trace the onset and resolution of inflammation compared with other acute proteins with longer half-lives (Vermeire et al., 2006). Additionally, medical therapy does not seem to influence CRP production in the hepatocytes; therefore, changes observed during treatment should be caused be the observed treatment on the existing disease (Henriksen et al., 2008). Under typical conditions, CRP is produced by hepatocytes in low quantities (≈ 1.0 mg/L) and is partially genetically regulated (Ford et al., 2003; Tall, 2004). Following an acute phase reaction, such as gut inflammation, CRP is rapidly produced, with potential for peak levels reaching between 350-400 mg/L. In cases of mild inflammation or viral infections, the concentration of CRP is typically observed between 10-40 mg/L.

As previously mentioned, CRP is upregulated in most inflammatory diseases, including IBD. However, since it may be affected by other non-IBD related phenomenon, the utility of

CRP in IBD is limited. Historically, a notable difference exists between the strong CRP responses in CD compared with the moderate, weak or absent CRP responses in UC, despite active inflammation (Vermeire et al., 2006).

Unfortunately, the correlation between CRP and disease activity in IBD has been inconsistent (Andre, Descos, Landais, & Fermanian, 1981; Boirivant et al., 1988; Brignola et al., 1986; Buckell et al., 1979; Fagan et al., 1982; Niederau et al., 1997). However, the more recent assessment of high-sensitivity C-reactive protein (hsCRP) in CD patients suggests a stronger association with disease activity. The assay for hsCRP measures the same serological CRP molecule, however, hsCRP can detect very small amounts in the blood that were previously below the detection limit (0.5-10 mg/L) ("CRP: Common Questions"; Sidoroff, Karikoski, Raivio, Savilahti, & Kolho, 2010). The assessment hsCRP was observed to be more effective (100% sensitivity/67% specificity) in differentiating between functional and new IBD diagnoses (Poullis et al., 2002). Additionally, serum levels of CRP (when an individual mounts a CRP response) are useful for assessing a patient's risk of relapse and response to medication (Van Assche et al., 2010; Vermeire et al., 2004). However, it's utility is limited in IBD as some patients do not mount a CRP response (Jones, Loftus, Panaccione, Chen, Peterson, Mcconnell, et al., 2008)

2.2.2 Fecal Markers

Although serum markers are useful in assessing active inflammation, they are non-specific and elevated in conditions unrelated to IBD. Ideally, a specific marker or set of markers of gastrointestinal inflammation would make endoscopy avoidable in various IBD related instances (i.e. diagnosis, monitoring therapy). The inflamed intestinal mucosa is populated with a larger number of neutrophils that are in direct contact with the fecal stream. Neutrophil derived

proteins, calprotectin and lactoferrin being the most widely studied, present the most ideal type of biomarkers in the study of gut inflammation (Abraham & Kane, 2012). Furthermore, although assumed, analyzing a fecal derived marker improves sample accessibility in this patient population.

Fecal Inflammatory Markers

Calprotectin, a calcium-binding protein discovered in 1980, is found in neutrophils, and to a lesser degree in monocytes and macrophages, and comprises a large portion of cytosolic (60%) and total (≈5%) protein of these neutrophils (Dale, Brandtzaeg, Fagerhol, & Scott, 1985; Fagerhol, Andersson, Naess-Andresen, Brandtzaeg, & Schjønsby, 1990; Røseth, Fagerhol, Aadland, & Schjønsby, 1992). Calprotectin plays a regulatory role in the inflammatory process, has bacteriostatic and fungistatic properties and can be measured in various biological fluids, including plasma, urine, and stool. Plasma calprotectin has been observed to increase between 5- and 40-fold in infectious and inflammatory conditions (Bunn, Bisset, Main, & Golden, 2001). Measurement in feces has proven advantageous to the study of gut inflammation, mainly due to the characteristics of neutrophils. Neutrophils migrate through the intestinal mucosa and into the lumen as the last step in their turnover (Røseth et al., 1992). Therefore, the presence of FC in human feces is directly proportional to neutrophil migration in the gastrointestinal tract (Vermeire et al., 2006). The concentration of FC is ~6 times that of normal plasma levels (Fagerberg, Lööf, Merzoug, Hansson, & Finkel, 2003). FC is an attractive clinical marker; it is resistant to colonic bacterial degradation and is evenly distributed and stable in stool for up to one-week at room temperature (Røseth et al., 1992). The release of FC is more than likely a result of cell disruption and death, a by-product of the inflammatory process, although it can also be actively secreted (Rammes et al., 1997; Voganatsi, Panyutich, Miyasaki, & Murthy, 2001).

FC has been consistently observed at increased concentrations in IBD, colorectal carcinoma, and nonsteroidal enteropathy patients (Kristinsson et al., 1998; Meling, Aabakken, Røseth, & Osnes, 1996; Røseth, Aadland, Jahnsen, & Raknerud, 1997; Røseth et al., 1992; Teahon, Roseth, Foster, & Bjarnason, 1997; Tibble et al., 2001; Tibble et al., 1999). A meta-analysis conducted by von Roon and colleagues (2007) found FC to be superior to serological markers (i.e. CRP, ESR) in its ability to diagnose IBD (von Roon et al., 2007). An exceptional meta-analysis by Van Rheenen and colleagues (2010) investigated the use of FC in a similar vein; however, the analysis only incorporated the diagnostic accuracy of FC studies with suspected cases of IBD (not IBS or healthy controls) and analyses that occurred prior to endoscopy. The pooled sensitivity and specificity rates of FC in this meta-analysis were 93% and 96%, respectively (van Rheenen et al., 2010). Burri and Beglinger (2012) provide an extensive review of the relationship between FC and endoscopic measures of disease activity. **Table 2.2** presents the studies investigating the correlation between FC and endoscopic activity in both UC and CD (Burri & Beglinger, 2012). FC displays modest to good correlation with endoscopic activity. In UC, the correlation is stronger with more objective measures of endoscopic scoring (i.e. Sutherland, Mayo). Differences in study design and type of endoscopic assessment are hypothesized to play a role in the range of observed correlations.

Table 2.3 Relationship between fecal calprotectin and endoscopic disease activity

Author	Patients/ No. of Endo.	Disease	Scoring	r
Bunn, SK (2003)	22/22	UC/CD	Saverymutt	0.75
D'Inca, R (2007)	46/46	UC	Mayo Score	0.51
D'Inca, R (2007)	31/31	CD	SES-CD	0.48
Langhorst, J (2008)	42/42	UC	RI	0.49
Langhorst, J (2008)	43/43	CD	SES-CD	0.35
Roseth, AG (1997)	62/64	UC	Mayo Score	0.57
Jones, J (2008)	164/164	CD	SES-CD	0.72
Schoepfer, AM (2010)	140/140	CD	CDEIS	0.75
Denis, MA (2007)	28/28	CD	CDEIS	ns
Xiang, JY (2008)	66/66	UC	Sutherland	0.87
Hanai, H (2004)	31/31	UC	Matts'	0.81
Aomatsu, T (2011)	17/17	UC	Matts'	0.84
Aomatsu, T (2011)	18/18	CD	SES-CD	0.76
Langhorst, J (2005)	31/31	UC	RI	0.51
Sipponen, T (2008a)	61/87	CD	SES-CD	0.64
Fagerburg, UL (2003)	39/39	UC/CD	Custom	0.52
Sipponen, T (2008b)	77/106	CD	CDEIS	0.73
Schoepfer, AM (2009)	134/134	UC	RI	0.83
Sipponen, T (2008c)	15/15	CD	CDEIS	0.83

Adapted from Burri and Beglinger (2012). **r**, correlation value, **UC**, Ulcerative colitis, **CD**, Crohn's disease, **SES-CD**, Simple Endoscopic Score for Crohn's disease, **RI**, Rachmilewitz index, **ns**, not significant.

The majority of IBD patients with clinically inactive disease seem to have some degree of residual mucosal inflammation (Saverymuttu, Hodgson, Chadwick, & Pepys, 1986). Interestingly, elevated FC levels have been detected in patients in clinical remission (Taina Sipponen & Kolho, 2010). In a seminal study investigating disease relapse, Tibble and colleagues (2000) found that within clinical remission, higher levels of FC were found among patients that experienced a relapse as opposed to patients staying in remission (Tibble et al., 2000). Since then, several studies have observed that FC levels predict relapse in IBD patients (Costa et al., 2005; D'Incà et al., 2007, 2008; Diamanti et al., 2008; Gisbert et al., 2009; Kallel et al., 2010; Sipponen & Kolho, 2010; Walkiewicz et al., 2008). Limited work has be conducted in

regards to FC and the response to therapy (Aadland & Fagerhol, 2002; Røseth, Aadland, & Grzyb, 2004). Although the studies are small with various designs, the general trend suggests FC may have a role in predicting response to therapy (Ho et al., 2009; Sipponen et al., 2008, 2010; Turner, Leach, et al., 2010; Turner, Mack, et al., 2010; Wagner, Peterson, Ridefelt, Sangfelt, & Carlson, 2008).

Lactoferrin (Lf), an iron-binding glycoprotein secreted by the majority of mucosal membranes, is a major component of the secondary granules in polymorphonuclear neutrophils (PMN) (Baveye, Elass, Mazurier, Spik, & Legrand, 1999; Guerrant et al., 1992; Levay & Viljoen, 1995). PMN cells play a crucial role in the acute primary inflammatory response (Kayazawa et al., 2002). Lf is released upon activation and degranulation of these cells (Guerrant et al., 1992; Hayakawa, Jin, Ko, Kitagawa, & Ishiguro, 2009). Lf possesses anti-microbial properties and is resistant to proteolysis in feces (Angriman et al., 2007).

Similar to FC, Lf has been observed to be very accurate in diagnosing IBD. In discriminating from IBS, Schoepfer and colleagues (2008) found that Lf was 91% specific. Overall accuracy for discrimination of IBS from patients with CD in remission (CDAI<150) was 90% for both Lf and FC (Schoepfer et al., 2008).

Additionally, elevated levels of fecal Lf are increased in active ulcerative UC and CD. Lf is also elevated in inactive IBD, above levels from IBS patients and healthy controls. Furthermore, fecal Lf concentration has a 93% correlation with measures of disease activity (Sugi, Saitoh, Hirata, & Katsu, 1996; van der Sluys Veer, Biemond, Verspaget, & Lamers, 1999; Walker et al., 2004). In additional studies, sensitivity of fecal Lf for IBD was 78% (95% confidence interval [CI], 69%–83%), and the specificity was 90% (95% CI, 83%– 96%), correlating well with endoscopic and histologic grading of disease activity (Kane et al., 2003;

Uchida et al., 1994). Furthermore, elevated fecal Lf was 100% specific in ruling out IBS (Kane et al., 2003). Finally, in 177 patients with active IBD, fecal Lf was significantly higher in those with active IBD compared with those with inactive disease, IBS patients, those with enteric infection, and healthy volunteers. The sensitivity and specificity of fecal Lf were 92% and 88%, respectively, for UC, and 92% and 80%, respectively, for CD (Dai, Liu, Zhao, Hu, & Ge, 2007).

In a study of 164 patients with CD, no significant associations between the CDAI scores and the fecal concentrations of Lf were found. However, Jones and colleagues (2008) found significant association between Lf concentration and endoscopic activity (Jones, Loftus, Panaccione, Chen, Peterson, McConnell, et al., 2008). As previously mentioned, Kane and colleagues (2003) found fecal Lf correlated well with endoscopic and histologic grading of disease activity in CD (Kane et al., 2003). Furthermore, Sipponen (2008) and colleagues evaluated endoscopic scores, through CDEIS, in 77 CD patients undergoing ileocolonoscopy. Lf correlated significantly with CDEIS (Spearman's r =0.773, $p<0.001$). Setting a fecal Lf cut-off value at 10 µg/g gave a sensitivity, specificity, PPV, and NPV of 66%, 92%, 94%, and 59%, respectively, in contrast to a CDAI value (>150) producing a sensitivity of 27%, specificity of 94%, of PPV 91%, and of NPV 40% to detect endoscopically active disease (Sipponen et al., 2008). Vieira and colleagues (2009) evaluated 78 patients presenting with IBD: 52 patient samples demonstrated inflammation based on histology. From these, 49 were Lf positive, with fecal Lf concentration correlating with disease activity, measured through the Mayo Disease Activity Index Values (Vieira et al., 2009). Masoodi and colleagues (2009) demonstrated the utility of Lf in diagnosing IBD, finding elevated Lf levels having 94% sensitivity and 100% specificity in diagnosing UC. Importantly, after treatment, fecal Lf significantly decreased along with disease activity, measured through Mayo scores (Masoodi et al., 2009). Gisbert, McNicholl,

and Gollomon (2009) provide an extensive review of many common questions pertaining to fecal Lf and IBD. Overall, Lf may be a helpful non-invasive tool to distinguish between IBD and functional disease, although it may not be as accurate as FC (Jones et al., 2008). Furthermore, the use of fecal Lf to assess disease activity and as a marker of therapeutic response in IBD holds promise.

Previous cohort studies in IBD exploring predictors of disease course have had limitations due to the heterogeneity and confounding effect of numerous subject and disease-related characteristics of the subjects within the sample population. When studying a population of patients with long-established disease, patients are subject to different medications, complications and surgeries, distorting the usefulness and calculation of biomarker concentrations and measures of disease activity. Thus, studying newly diagnosed patients provides a more homogenous sample population and limits confounders. **Table 2.4** summarizes the utility of fecal biomarkers in IBD.

Table 2.4 Summary of fecal biomarker utility

Type	Calprotectin	Lactoferrin
Distinguish IBD vs. IBS	yes	yes
Distinguish CD vs UC	no	no
Active disease vs. remission	yes	yes
Sensitivity	78 – 100%	66 – 80%
Specificity	44 – 100%	67 – 100%
Assess mucosal healing	yes	Yes
Correlation coefficient (r)	0.48 - 0.83	0.19 – 0.87
Predict relapse	yes	yes
Predict response to treatment	yes	yes

Adapted from Lewis (2011). **UC**, ulcerative colitis, **CD**, Crohn's disease, **IBD**, Inflammatory Bowel Disease, **IBS**, irritable bowel syndrome.

Short Chain Fatty Acids

Short-chain fatty acids (SCFA) are by-products of anaerobic fermentation of dietary fibre and sugars in the colon (Flint, Duncan, Scott, & Louis, 2015). Acetate, propionate, and butyrate are the major SCFA. Colonic epithelial cells (colonocytes) rapidly absorb and oxidize these SCFA, using butyrate as their primary source of energy (Hamer et al., 2008). Butyrate also plays a major role in the physiology of the colonic mucosa. Butyrate metabolism was first shown to be impaired in IBD by Roediger (Roediger, 1980). In this disease state butyrate is underutilized due to a decrease in its β-oxidation. Cummings (1981) reported that the major SCFA are found in human feces in a ratio as 60:24:16 of acetic : propionic : butyric acid, respectively. Although its role in the etiology of IBD is in question, butyric acid has a well-recognized anti-inflammatory potential in addition to influencing the colonic defence barrier and intestinal wall permeability (Hamer et al., 2008; Segain et al., 2000; Van Immerseel et al., 2010). Fecal concentrations of SCFA and lactate, collectively termed organic acids, have been observed to be positively correlated with clinical measures of disease activity using the Truelove-Witts criteria and CDAI (Mortensen & Clausen, 1996; Roediger et al., 1982; Treem, Ahsan, Shoup, & Hyams, 1994).

Conversely, the concentration of organic acids in relation to disease activity, in both active and inactive states of IBD have been inconsistent (Hove & Mortensen, 1995; Hove, Nordgaard-Andersen, & Mortensen, 1994).

Recently, Huda-Faujan et al. (2010) investigated the concentration of fecal SCFA in IBD patients. In the IBD patients the level of acetic acid, 162.0 µmol/g wet feces, butyric acid, 86.9 µmol/g wet feces, and propionic acid, 65.6 µmol/g wet feces, was significantly lower when compared with that of healthy individuals, 209.7, 176.0, and 93.3 µmol/g wet feces, respectively. It should be noted that the study was conducted in Malaysia where the diet consists mainly of starch. The study was also limited by a very small sample size (n=8). Overall, there is contradictory data in the observation of organic acids in IBD. Furthermore, limited studies have assessed organic acids with endoscopic disease activity data; in addition to clinical disease activity has never been performed.

2.3 Causation and Environmental Determinants in IBD

2.3.1 Environmental Determinants of IBD: The pathogenesis and aetiology of IBD, although not completely understood, is hypothesized to develop as a result of the interaction between three factors: genetic susceptibility, environmental exposures and host immune response (Cashman & Shanahan, 2003). Different genes have been implicated in the pathogenesis of IBD. The influence of environmental factors on disease onset and progression has been suggested by low concordance rates in identical (monozygotic) twins with IBD (CD [B50%] and UC [B10%]) (Halfvarson, Bodin, Tysk, Lindberg, & Järnerot, 2003). Additionally, population-based data points to an increasing incidence of IBD in migrant populations highlighting the importance of environmental factors in the pathogenesis of IBD (Cashman & Shanahan, 2003). Furthermore, the discovery that genetically engineered animal models of IBD do not develop colitis under germ-free conditions displays the importance of the intestinal microflora as an environmental factor that influence IBD pathogenesis (Taurog et al., 1994).

These environmental influences may occur at both the local microenvironment (enteric microflora) and the nutritional environment (Shanahan, 2001). The influence of hygiene and nutritional factors, in combination with unfavourable alterations of the gut microbiota, on the regulation of inflammation has designated them as "prime environmental triggers for the development and modification of lifestyle-related chronic diseases", not solely IBD (Haller, 2010).

2.3.1.1 Smoking

The extensive study of the relationship between environmental factors and IBD has resulted in widespread connections, none of that are more puzzling than the effect of smoking on the disease. Generally, it has been observed that the smoking of cigarettes is a risk factor for patients with CD, whereas non-smoking is a risk factor of UC (Harries, Baird, & Rhodes, 1982; Reif, Klein, Arber, & Gilat, 1995; Somerville, Logan, Edmond, & Langman, 1984). Smoking has been shown to be an independent risk factor for clinical, surgical and endoscopic recurrence in CD, with further influence on disease activity after surgery. Interestingly, although the exact component of tobacco that produces this effect is unknown, the addition of nicotine to conventional medical therapy was observed to improve symptoms in mild to moderate UC (Pullan et al., 1994). Mechanistically, this association is plausible; nicotine has been shown to have a modulatory effect on immune responses *in vitro* (Madretsma et al., 1996). This important observation may highlight specific differences between the pathogenesis of CD and UC.

2.3.1.2 Microbiota and Bacterial Composition

The increased incidence of IBD may be due to local phenomena within the intestinal microenvironment coupled with genetic polymorphisms and harmful environmental exposures prevalent in westernized lifestyles and industrialized environments. In particular, diet has been implicated as an important modifiable risk factor in the development of chronic digestive diseases (“Chronic Disease,” n.d.). Human health is highly dependent on the relationship between the gut and the resident microbial flora it contains. Changes to this microbial community can stimulate the immune system, create dysfunction in gut epithelial cells, and increase the permeability in the gastrointestinal tract, leading to chronic inflammation of the gastrointestinal tract (Sartor, 2008). Healthy individuals have a microbial ecology that is

characterized by a stable dominant microbiota, referred to as "normobiosis", that exhibit high biodiversity and resilience (Marteau, 2009; Roberfroid et al., 2010). In contrast, the microbiota in IBD patients display decreased diversity, a state known as "dysbiosis", where one or a select few potentially harmful micro-organisisms are dominnant (Kelly & Mulder, 2012; Roberfroid et al., 2010). Reduced diversity may create innappropriate mucosal cellular responses, that may be responsible for the prolonged inflammatory response observed in intestinal disease (Murphy, Kwon, & Boone, 2012). Microbiota and fungi increase in concentration and complexity throughout the gastrointestinal tract, from 10^2-10^3 aerobic organisms/g of luminal contents in the stomach and duodenum to 10^{11}-10^{12} bacteria/g of contents in the colon and cecum (**Figure 2.2**). It has been shown that greater than 99% of gut microbiota is composed of species within four of the following bacterial phyla: Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Eckburg & Relman, 2007; Frank et al., 2007).

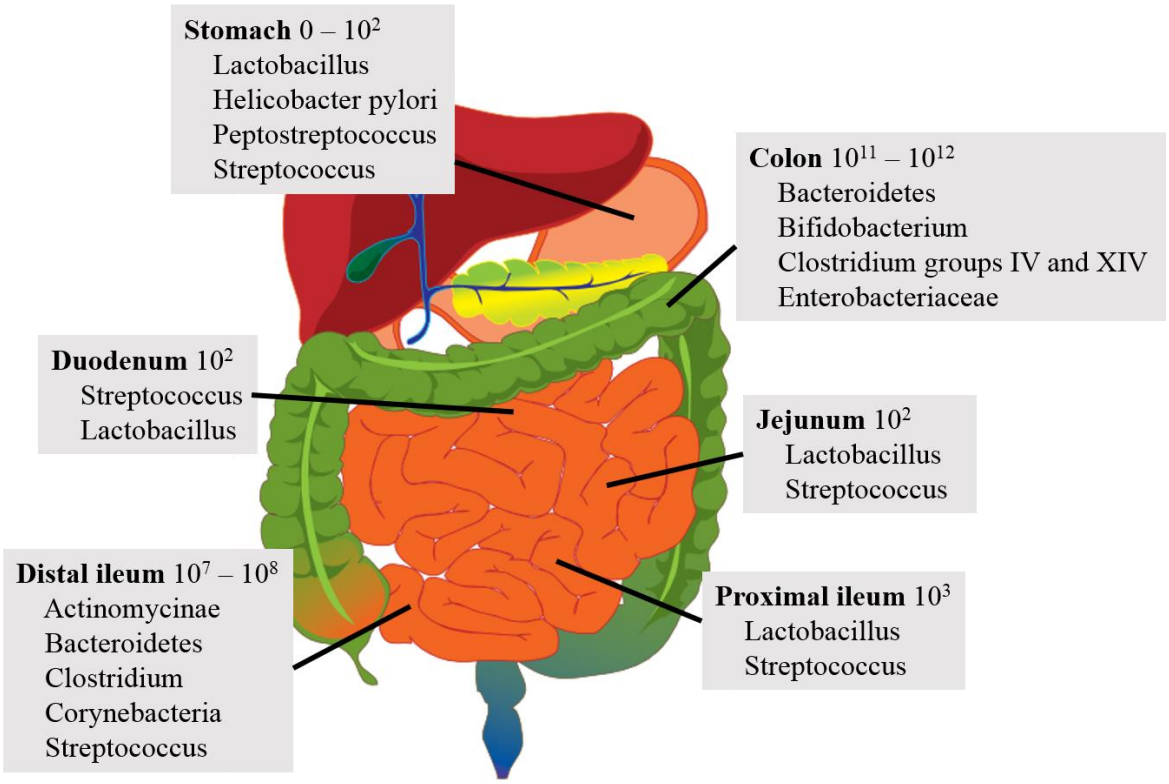

Figure 2.2 Composition and luminal concentrations of dominant microbial species (listed in alphabetical order) in the gastrointestinal track (adapted from Sartor, 2008).

The following observations implicate the resident microbiota in the pathogenesis of IBD. First, from animal models of IBD, interleukin-10 (IL-10) deficient mice do not develop intestinal inflammation under germ-free conditions (Sellon et al., 1998). However, these animals will develeop intestinal inflammation when they are introduced to non-sterile conditions or artifically colonized with microbiota. In humans, a similar concept demonstrates the significance of the mictobiota in inflammation. The use of an ileostomy, that diverts the fecal stream from the active inflammation, results in remission in 65% of patients, while reversal of this procedure results in disease relapse in 60% of patients. Therefore, this luminal content may play a role in active inflammation (Fasoli, Kettlewell, Mortensen, & Jewell, 1990). However, not all gut microbiota have a negative impact on intestinal inflammation. Some bacteria, in both animal models and

IBD patients, have been shown to decrease abnormal intestinal permeability (Garcia Vilela et al., 2008; Miyauchi, Morita, & Tanabe, 2009). The decrease in intestinal permeability is thought to reduce the exposure of the mucosal immune system to the gut microbiota (Roberfroid et al., 2010). Additionally, some strains of bacteria, particularly bifidobacteria, up-regulate the production of IL-10 by dendritic cells, a response shown to be therapeutic in animal models of IBD (Hart et al., 2008; Lindsay & Hodgson, 2001). Therefore, the use of anti- and probiotics in the management of IBD is logical, and extensive reviews have been completed elsewhere (Hedin, Whelan, & Lindsay, 2007; Sartor, 2008). Alternatively, fecal microbiota transplants (FMT), which transfer gut microbiota from healthy donors by way of stool infusion, have been used occasionally in IBD (Borody & Khoruts, 2012). A recent systematic review found 17 studies (n=41) using FMT therapy in IBD (Anderson, Edney, & Whelan, 2012). The majority of patients undergoing FMT saw improvements in IBD symptoms, with remission from disease and cessation of IBD therapy (Anderson et al., 2012).

Recently, the use of 16S rRNA sequencing has been employed to investigate different microbial populations in CD and UC patients and healthy individuals. In a study by Frank and colleagues (2007), a specific subset of CD and UC patients were observed to have a significantly different microbial profile than controls and other IBD patients of a different subset. This subset was observed to have a depletion of commensal bacteria, with a tenfold lower bacterial load, that affected both major classes of commensal phyla, Firmicutes and Bacteroidetes. Changes to the gut microflora in human IBD are displayed in **Table 2.5**.

Table 2.5 Microbial flora change in IBD

Decreased abundance	Increased abundance	Total Bacterial Amount	Sample Origin
Bacteroidetes including *Bacteriodes thetaiomatciron*[1]	Proteobacteria including Enterobacteriae (relative, not absolute)	Decreased in recent 16S rRNA studies [1,4]	Intestinal
Clostridia class of Firmicutes including *Faecalibaceterium praunsnitzii* [1-2, 4-6] and butyrate-producing spp [1].	Bacilli class of Firmicutes[1]	Increase in DGGE and FISH studies [3,9]	
Reduced diversity [5-7].	Increase in mucosal adherent bacteria (particularly in adjacent uninflamed		

Adapted from Kaser (2010). **rRNA**, ribosomal ribonucleic acid, **DGGE**, degraded gradient gel electrophoresis, **FISH**, fluorescence in situ hybridization. [1](Frank et al., 2007); [2](Sokol et al., 2008); [3](Swidsinski et al., 2002); [4](Baumgart et al., 2007); [5](Manichanh et al., 2006); [6](Gophna, Sommerfeld, Gophna, Doolittle, & Veldhuyzen van Zanten, 2006); [7](Ott et al., 2004); [8](Martinez-Medina, Aldeguer, Gonzalez-Huix, Acero, & Garcia-Gil, 2006); [9](Bibiloni, Mangold, Madsen, Fedorak, & Tannock, 2006).

Additionally, specific dietary components, such as prebiotics, have the potential to alter this microbial community. A prebiotic is defined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" (Gibson, Probert, Loo, Rastall, & Roberfroid, 2004). The further study of resident microbiota, and the components that may influence it, could identify alternative methods to managing intestinal inflammation (Roberfroid et al., 2010)

2.3.1.3 Diet

The relationship between nutrition and IBD can be divided into three main subcategories: 1) specific dietary components in the pathogenesis of the disease, 2) malnutrition or nutritional deficiencies in the course of disease, and 3) nutritional therapy in response to the disease

(Geerling, Stockbrügger, & Brummer, 1999). Maconi et al. (2010) explains the difficulty in elucidating a clear relationship between diet and IBD because of the "the possibility that early symptoms of the disease may lead to a modification in dietary habits and the inability of the patients to accurately remember their diet before the onset of symptoms". The prevalence of nutrition deficiencies and malnutrition in IBD and the effect on disease progression are well documented (Han, Burke, Baldassano, Rombeau, & Lichtenstein, 1999; Vagianos, Bector, McConnell, & Bernstein, 2007). However, the role of an optimal diet or dietary recommendations during this period is less clear.

No standard diet is given to newly diagnosed patients with IBD (Steinhart, 2012). When the disease is under control, a patient is advised to follow the healthy eating habits such as found in Health Canada's "Eating Well with Canada's Food Guide" ("Eating Well with Canada's Food Guide," 2007). However, when the disease is active, dietary modifications can be made to normalize bowel function, reduce gastrointestinal symptoms, maintain or improve hydration and electrolyte status, and to avoid malnutrition. Important to remember is that these experiences are patient specific. When discussing the role dietary management plays in CD, Russell (1991), states the merit of "lactose restriction, low-fat diets and low-residue diets,... in specifically indicated clinical situations". In the past, low-residue or low-fibre diets (sometimes used interchangeably) were often recommended to minimize food residue, in turn limiting gastrointestinal distress (Hosoi, 1928). However, the benefits of fibre (i.e. production of SCFA) warranted further study of this relationship. Dietary fibre lessens the features of colitis in mouse models, but the results in human studies have been conflicting (Cabré & Domènech, 2012; Fernandez-Banares et al., 1999; Nanau & Neuman, 2012; Ritchie, Wadsworth, Lennard-Jones, & Rogers, 1987). Furthermore, IBD patients modify their diets as they experience gastrointestinal

symptoms even before diagnosis of the disease. In a case-control study of 83 new cases (within 12 months of diagnosis) of IBD (41 UC, 42 CD), Maconi and colleagues observed that 38.6 % of patients made intentional changes to their diet, due to the presence of symptoms (Maconi et al., 2010). Changes included the reduction of fat and calorie intake (12 patients), or the reduction or complete elimination of dietary fibre (18 patients) and milk or cheese (9 patients). Thus, it is prudent that a patient suffering from IBD discusses dietary modifications with a physician or registered dietitian in order to avoid unnecessary and potentially harmful dietary restrictions.

2.3.2 Specific Nutritional Components of Diet:

2.3.2.1 Nutritional Deficiency

The potential for nutritional deficiency in IBD is well documented, largely due to the use of objective markers of nutritional status and a relatively homogeneous patient population (active disease, inpatients) (Geerling, Badart-Smook, Stockbrügger, & Brummer, 2000; Han et al., 1999; Vagianos et al., 2007). Particularly, the lack of zinc in CD patients has been observed, leading to immune dysfunction (Ainley, Cason, Slavin, Wolstencroft, & Thompson, 1991). Generally, it is easier to observe how the disease affects the diet, but the reverse, how diet may affect the disease process is difficult to elucidate. Additionally, active intestinal disease can induce decreased food intake, coupled with an alteration in the normal physiology of digestion and absorption, leading to depletion of macro- and micronutrients. Protein loss is a longstanding observation in IBD (Kirsner & Koch, 2006).

2.3.2.2 Dietary Protein and Meat Consumption

The observed increase in the incidence of IBD, in both developed and developing countries, has urged researchers to evaluate population models in the search for a specific dietary cause. Shoda and colleagues (1996) observed the association of increase dietary protein intake

and incidence of CD in Japan (Shoda, Matsueda, Yamato, & Umeda, 1996). This dietary trend was also observed in Belgium, with a reported 50% increase of per capita meat consumption per year, between the 1950s and 1978 (Larsen, 2003). Additionally, a few, but not all, retrospective case control studies have shown a higher consumption of meat and fish in CD patients compared with controls (Abubakar et al., 2007; Sakamoto et al., 2005). Conversely, a large prospective study in multiple European centres failed to find any association between UC incidence and the consumption of a particular macronutrient (Hart et al., 2008). A recent cohort of French women observed the development of IBD was associated with total protein (hazard ratio for third vs first tertile, 3.31; 95% CI, 1.41-7.77) and animal protein intake (hazard ratio, 3.03; 95% CI, 1.45-6.34) (Jantchou, Morois, Clavel-Chapelon, Boutron-Ruault, & Carbonnel, 2010). The relationship between dietary protein and meat consumption is unclear. However, the metabolic by-products of animal protein, metabolized in the colonic lumen by gut flora, have been hypothesized to play a role in IBD pathophysiology.

2.3.2.3 Dietary Carbohydrates

Carbohydrates and Chronic Disease

Carbohydrates are the principal dietary constituents that influence postprandial glycemia (the rise in blood sugar after a meal) and insulin secretion (Brand-Miller, 2004). Many have hypothesized that increased postprandial glycemia has a principal role in the etiology of many chronic diseases, a theory that continues to be investigated. Proponents of this hypothesis claim that “higher postprandial glycemia is a universal mechanism for disease progression” as stated by Barclay et al. (2008) in conclusion to their meta-analysis of observational studies investigating the relationship between postprandial glycemia and chronic disease risk. This meta-analysis included 37 prospective cohorts (a total of 40,129 incident cases) investigating the

relationship between glycemic index (GI), glycemic load (GL) and chronic disease risk. In this meta-analysis, Barclay et al. (2008) found that diets with a high GI or GL independently increased the risk of type 2 diabetes (GI RR 1.40; GL RR 1.27), heart disease (GI RR1.25), gallbladder disease (GI RR1.26; GL RR 1.41), breast cancer (GI RR 1.08), and all diseases studied combined (GI RR 1.14; GL RR 1.09). This study was strengthened by the number of incident cases available in the analysis, but limited in the fact the patient population was 90 percent female and that no study validated the assessment of GI and GL against another dietary collection method. Nonetheless, the role postprandial glycemia may play in chronic disease remains a contentious subject in clinical nutrition (Barclay et al., 2008).

Traditionally, carbohydrates in the diet have been classified by chemical structure (mono, di, oligo-, and polysaccharides, starch and fibre) (Chen, Shaw, & Moyer-Mileur, 2010; Riccardi, Rivellese, & Giacco, 2008). However, classifying carbohydrates by chemical structure dismisses their impact on a physiological level - carbohydrates have variable impact on glucose and insulin levels, in addition to an effect on satiety and gastric emptying (Chen et al., 2010).

Role of Dietary Carbohydrates in IBD

The most consistent observation in the study of nutrition and IBD (mostly CD and limited UC) has been an increase in sugar consumption in the diet (Cashman & Shanahan, 2003; Geerling, Stockbrügger, et al., 1999). This association first appeared in observational studies in the mid-1970s, showing significantly increased consumption of refined carbohydrates (large quantities of sweets, prior to diagnosis) in CD patients compared with controls (Martini & Brandes, 1976; Miller, Fervers, Rohbeck, & Strohmeyer, 1976).

This association prompted several clinical trials testing the therapeutic effect of a low-

refined carbohydrate, high-fibre diet in IBD. These studies produced conflicting results (Jones et al., 1985; Lorenz-Meyer et al., 1996; Ritchie, Wadsworth, Lennard-Jones, & Rogers, 1987). However, these studies contain various methodological limitations and flaws. The study conducted by Ritchie and colleagues (1987) was a large-scale multi-centre trial investigating the effect of a low-refined carbohydrate and high-fibre diet in CD. In the study, 162 patients were counselled to either continue on with consuming their traditional diet (i.e. high in refined carbohydrate and low in fibre) or counselled to adhere to the interventional diet (low in refined carbohydrate and high in fibre). Results showed good dietary compliance in both groups; however, the diet had no effect on disease activity. The major criticism of this study (Cashman & Shanahan, 2003) is that the study actually tested the use of dietary counselling for patients rather than the effect of the specific diet (Husain & Korzenik, 1998). Also interesting, Ritchie and colleagues found their results "disappointing", but nonetheless emphasized that "a therapeutic response would have been more likely among patients with active disease" (Ritchie et al., 1987). However, a study in patients with active disease would not be conducted because the primary medical therapy would confound the results (Ritchie et al., 1987). The study by Lorenz-Meyer et al. (1996) studied the effect of a reduced carbohydrate diet, not reduced refined-carbohydrate diet. The rationale for this strategy was that some refined carbohydrate sources are contained in the elemental diets shown to be "very effective in treating an acute flare-up in Crohn's disease". However, remission rates between patients on the two diets were identical after 1-year. Overall, the major issues with these dietary interventions were associated with data captured too far from diagnosis and the heterogeneity of the study population (Geerling, Houwelingen, Badart-Smook, Stockbrügger, & Brummer, 1999). However, several studies that have assessed dietary intake in newly diagnosed patients with CD, have demonstrated an increased consumption of sugar by CD

patients than that of controls (Geerling et al., 2000; Geerling & Houwelingen, et al., 1999; Mayberry et al., 1981). Geerling et al. (2000) further investigated the relationship between carbohydrate intake and disease activity in CD. Patients with high disease activity (CDAI >150) showed a significantly ($P<0.025$) higher total carbohydrate intake (56.4% ± 5.4) (% of daily energy intake) than CD patients in remission (48.7% ± 5.8). These results have implicated another limitation to this type of observatory study: a greater consumption of carbohydrates may be a result of the disease rather than the cause. In their review of consumption of sugar and CD, Riordan, Ruxton and Hunter (1998) suggest that the increase in sugar consumption may be a consequence of disease. Riordan et al. (1998) suggested this can be explained by the dietary advice to adhere to a low-fibre diet (less likely to produce symptoms) and that sugar containing foods are more palatable.

Glycemic Index and Glycemic Load

In an effort to quantify the glycemic response to carbohydrate-containing foods, the GI was introduced in 1981 (Jenkins et al., 1981). The GI value of a food is determined by measuring the glycemic response of a fixed amount of available carbohydrate in test food against the same amount of a standard food (glucose, white bread) in the same subject. This GI value is then quantified as the area under the blood glucose curve as a percentage of the standard (Jenkins et al, 2002). Therefore, as Dickinson and Brand-Miller (2005) describe, a lower GI suggests a slower digestion and absorption of glucose from foods and, therefore, is reflective of the quality of carbohydrate found in the diet (Hu, Block, Sternfeld, & Sowers, 2009). Foods can be classified by GI as low, moderate and high according to their glycemic response (as shown in **Table 2.6**). To quantify the quality *and* quantity of carbohydrates in the diet researchers have turned to a measure called the glycemic load (GL). The glycemic load of a food is the product of

the glycemic index and the amount of carbohydrate contained in the food. This value has direct physiologic meaning, as stated by Liu and colleagues (2001), because each unit can be interpreted as the equivalent of 1 g of carbohydrate from white bread. Liu et al. (2001) continues on to explain the utility of the GL as a tool to avoid categorizing a food as "good" or "bad" based solely on GI. For example, the GI of a carrot has been reported as 131 compared to bread, however, the GL for carrots is small because the amount of carbohydrates in one serving is small (7 g). In order to produce an incremental glycemic response 1.31 times that of 100 g of test standard, one would need to consume 700 grams of carrots (Liu et al., 2001) . Foster-Powell, Holt and Brand-Miller (2002) have compiled an international table of GI and GL values.

Table 2.6 Sample list of glycemic index values

High GI (>70)	Moderate GI (56-69)	Low GI (<55)
White bread	Brown rice	Barley
High sugar cereal	Banana, grape	Milk, yogurt
Bagel	Ice cream	Beans, chickpeas
Pretzels	Corn tortilla	Tree fruit
Hard candy	Spaghetti	Tomato
Russet potatoes	Corn, peas	Apple
Carrots	Whole wheat bread	Chocolate
Pancakes/waffles	Red potatoes	Peanuts
Glucose/sucrose	Lactose	Orange
Sports drinks	Soups	Fructose
Rice	Pizza	Non-starchy vegetable

Adapted from Chen (2010). **GI,** glycemic index.

In recent years, the values of GI and GL in the human diet have increased, mainly due to increases in carbohydrate consumption and advances in food processing (Ludwig, 2002). The GI and GL are important tools in nutrition research because they directly measure the postprandial glucose responses. The presence of excess postprandial blood glucose has been implicated as a developmental risk factor for cardiovascular disease and diabetes in prospective observational studies (Beulens et al., 2007; Schulze et al., 2004).

Glycemic Index, Glycemic Load, and Inflammation in IBD

As previously described, selected non-specific serological markers of inflammation, such as CRP, have been shown to be elevated and positively correlated with endoscopic disease activity scores in patients with CD. Interestingly, there is a potential association between dietary carbohydrates and acute phase proteins, such as CRP. In an investigation of 244 healthy women, Liu and colleagues (Liu et al., 2002) observed a strong and statistically significant positive association between dietary GL and plasma hsCRP. Furthermore, the observation of diets low in GL or GI have been shown to significantly lower the concentration of hsCRP in healthy overweight adults and type II diabetics (Nilsson, Ostman, Granfeldt, & Björck, 2008; Pittas,

Joseph, & Greenberg, 2004). These observations have led to the understanding that a diet with a "high intake of rapidly digested and absorbed carbohydrates may worsen the inflammatory process", as shown by the increases in markers of systemic inflammation (Hu et al., 2006; Qi & Hu, 2007). Oxidative stress may be the underlying physiological process responsible for these differences (Ludwig, 2002). Mechanistically, hyperglycemia causes an overproduction of superoxide anions by the mitochondrial electron transport chain that creates reactive oxygen species (ROS) and, therefore, contributes to oxidative stress (Brownlee, 2005). This theory has displayed merit; subjects who followed a low GI diet had a significant increase in total antioxidant capacity (Botero et al., 2009). The impact of GI and GL on inflammatory markers suggests that dietary carbohydrate is a principal contributor in any disease where inflammation or oxidative stress is a factor, such as IBD (Buyken et al., 2010; Mirrahimi et al., 2014).

Rationale for investigation of GI and GL in IBD

The quality and quantity of carbohydrates in the diet may play a role in the disease course of IBD. Furthermore, research investigating the role of nutrition in IBD when analyzing the endoscopic scores of disease is lacking. Lastly, the measures of GI and GL have never been assessed in IBD. There is a need for more research that investigates sugar consumption and its impact of IBD course and disease activity (Cashman & Shanahan, 2003).

The major weakness in many observational studies investigating influence of diet in IBD is they capture dietary information too far from diagnosis and, therefore, introduce a large amount of recall bias into the study. Studying diet in a newly diagnosed cohort of IBD patients would limit the confounding experienced during the study of heterogeneous (disease duration, disease behaviour, medical therapy, surgical history and altered gastrointestinal anatomy) IBD patient populations.

FODMAPs

Gibson and Shepherd (2005) have proposed a potential etiological dietary factor that may impact intestinal permeability. Due to their influence on osmotic activity and fermentation, the consumption of poorly absorbed, short-chain carbohydrates (Fermentable Oligo-, Di- and Monosaccharides and Polyols. "FODMAPs") have been targeted in dietary strategies to minimize functional symptoms (Barrett & Gibson, 2010). Elimination of FODMAPs containing foods may minimize functional symptoms in IBD patients, however, as Lomer (Lomer M C E, 2011) highlights, "it can be difficult to distinguish between whether symptoms are functional in nature or due to the ongoing inflammatory process". The elimination of functional symptoms may increase patient quality of life (QOL), which has been shown in mild to moderate chronic constipation and IBS, although the impact on the organic, inflammatory aspect of IBD is largely unknown (Rao, Yu, & Fedewa, 2015).

2.3.3 Methodological Design:

2.3.3.1 Disease Onset, Functional vs. Organic Disease

A clear relationship between diet and IBD is made even more difficult to ascertain due to a diagnostic bias. The time-line between environmental exposures, the onset of symptoms, the diagnosis of the disease and the introduction to an observational study all complicates the capture of dietary exposure (Molodecky, Panaccione, Ghosh, Barkema, Kaplan, et al., 2011). Furthermore, evidence showing that a patient will change their diet upon the appearance of symptoms complicates the study further (Maconi et al., 2010). Additionally, changes in dietary intake that may alleviate functional symptoms of disease may not be reflective of the ongoing inflammatory process (Issa & Saeian, 2011). As previously discussed, functional symptoms,

such as abdominal pain and diarrhea, can be present in both organic and functional intestinal conditions. Indices that evaluate these subjective symptoms must be used with caution.

2.3.3.2 Study Design and Outcomes

Historically, the study of diet in IBD study has relied on case-control studies gathering nutritional parameters between patients and controls. Rarely, does the study design measure the impact of intervention on disease activity. Disease activity can be assessed in IBD using clinical disease activity indices, endoscopic indices, serum serological markers, fecal markers and miscellaneous tests (Desai et al., 2007). The use of clinical scoring is partially subjective, as patients assess some parameters, and only give an indirect measure of disease activity (Desai et al., 2007). Endoscopic scoring is accurate; however, it is expensive and invasive. Recently, there has been a push to identify surrogate serological and fecal biomarkers. In the context of IBD, biomarkers may be used to assess disease activity, severity, and predict the course of the disease with low expense, high reliability and comparative ease to other assessments (Desai et al., 2007). In regards to nutrition interventions, the use of biomarkers may provide a more accurate way to measure impact on intestinal inflammation (Kuhnle, 2012). The disease process found in IBD is driven by active gut inflammation that is associated with an acute phase reaction and the migration of leukocytes to the gut (Langhorst et al., 2008). Therefore, the use of specific biomarkers, reflective of active gut inflammation, as a study outcome may give a better indication of the impact of a nutritional intervention of IBD.

CHAPTER 3

OVERVIEW OF EXPERIMENTAL DESIGN

The hypotheses outlined in Chapter Two were tested using the following experimental designs (**Figure 3.1**):

In **Chapter 4,** a previous dataset was examined from a study conducted at two tertiary care centres between 2004 and 2006. Overall, one hundred and sixty-four (n=164) patients were observed, with biomarker, endoscopic and clinical measures of disease activity being collected. This data was used to evaluate the relationship between biomarkers and endoscopic disease (Hypothesis **4-1**). We postulated that a model including biomarkers would outperform the CDAI, a conventional scoring system for disease activity (Hypothesis **4-2**).

In **Chapter 5**, the role of biomarkers in a longitudinal, inception cohort of newly diagnosed IBD patients was studied. The initial target for recruitment was 75 patients based on statistical power calculations, but was reduced to a target goal of 50 after 21 subjects were recruited in the first two years. Therefore, the scope of this study changed from an inception cohort to a pilot study, focusing on a descriptive analysis of our patient population. In total, 42 patients were recruited to the study between 2010 and 2015. We collected data on biomarkers (hsCRP, FC, Lf) and disease activity (endoscopic and clinical scores) to assess the relationship between markers and disease status (Hypothesis **5-1**). These data were also used to evaluate if biomarkers could predict response through repeated measurements (160 total observations) (Hypothesis **5-2**). Dietary intake was assessed through a validated Food Frequency Questionnaire (FFQ). Food groups were compared with reference standards to assess adequacy (Hypothesis **5-3**). Furthermore, measures of carbohydrate quality, GI and GL were calculated and correlated with fecal biomarkers (Hypothesis **5-4**).

In **Chapter 6**, FC was evaluated in healthy pregnant patients and pregnant patients with IBD. The aim was to recruit fifty (50) patients in this healthy cohort and test FC throughout pregnancy to establish healthy reference values (Hypothesis **6-1)**. Furthermore, ten (10) pregnant patients suffering from IBD were recruited to assess the relationship between FC and disease activity during this time (Hypothesis **6-2)**.

Lastly, in **Chapter 7,** a smaller subset of patients from our pilot cohort was used to observe fecal microbiota and SCFA in newly diagnosed IBD patients. The sample size was smaller (n=15) than the full cohort, as this project started after initial recruitment and aspects of sample collection were altered. As previously discussed, since measures of endoscopic and clinical disease activity were collected we hypothesized fecal SCFA would decrease with disease severity (Hypothesis **7-1).** Specifically, it was postulated that the concentration of fecal butyric acid would increase with the presence of the *Faecalibacterium prausnitzii* strain (Hypothesis **7-2).**

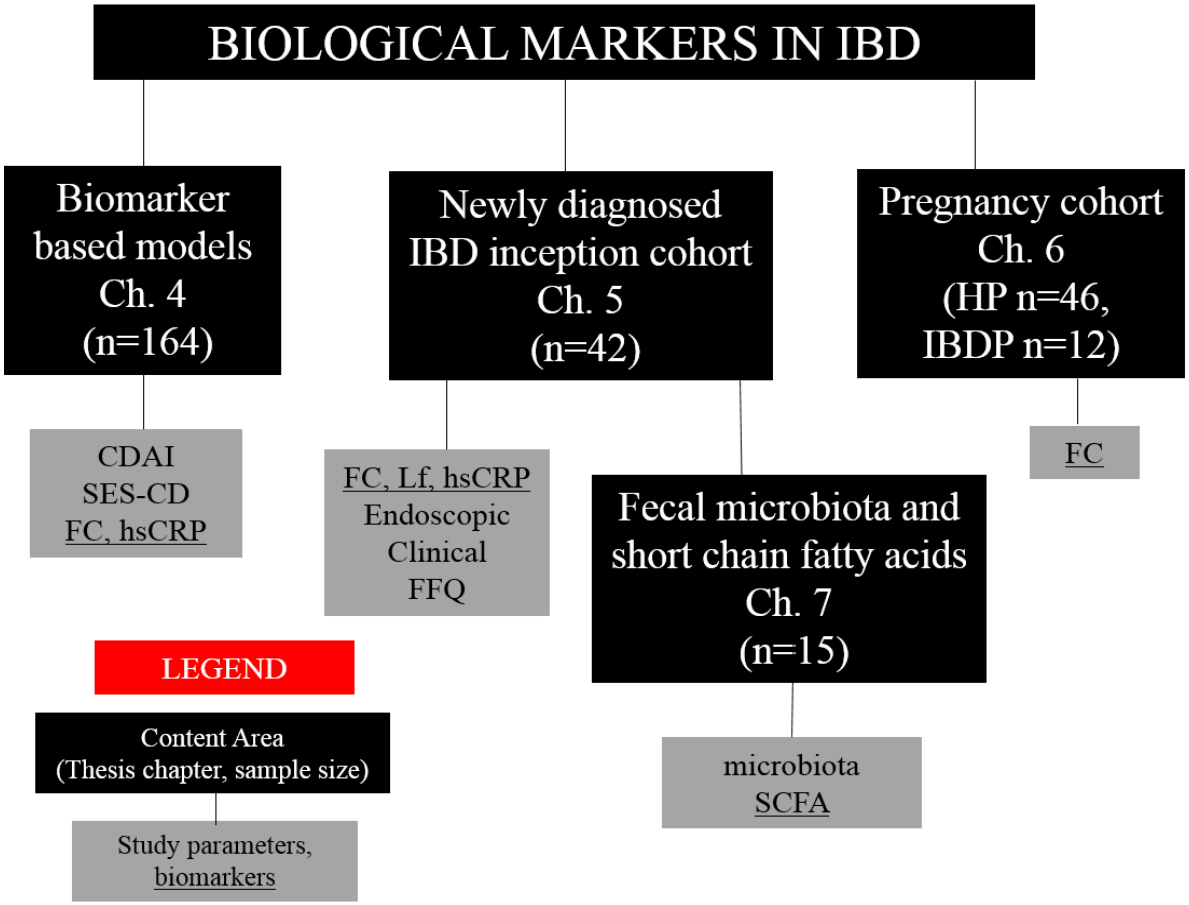

Figure 3.1 Biomarkers in IBD experimental design. **CDAI,** Crohn's disease Activity Index, **SES-CD**, Simple endoscopic score in Crohn's disease, **FC**, Calprotectin**, hsCRP**, high sensitivity C-reactive protein, **Lf,** lactoferrin, **FFQ,** food frequency questionnaire, **SCFA**, short chain fatty acids. **HP**, healthy pregnancy, **IBDP**, inflammatory bowel disease pregnancy

CHAPTER 4

BIOMARKER-BASED MODELS OUTPERFORM TRADITIONAL PATIENT REPORTED OUTCOME-BASED SCORES IN PREDICTING ENDOSCOPIC INFLAMMATORY DISEASE ACTIVITY

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Not yet published

The CDAI, a composite disease activity score comprised of patient and laboratory scores, has known limitations for accurately measuring inflammatory disease burden, but is still a widely used clinical tool. A PRO is any report or score supplied solely by the patient about a health condition or treatment. Although expensive and invasive, endoscopy is the gold standard for measuring inflammatory disease activity in IBD. In the future, there will likely be a move toward the use of a pure-PRO as a co-primary endpoint with endoscopy as the outcome measure of interest in clinical trials of novel therapeutics for the treatment of IBD. However, non-invasive markers of inflammation, such as the biomarkers discussed at length in this thesis, could prove more useful in the assessment and monitoring of disease activity. The objective of this study was to analyze the accuracy of individual components of the CDAI (both PRO- and lab-based), biomarkers and other variables of interest in predicting endoscopic disease activity, and to use the findings to create a new composite score.

Author role: I completed the data-mining, data entry and manuscript preparation and writing for this chapter.

Biomarker-based models Outperform Traditional Patient Reported Outcome-based Scores in Predicting Endoscopic Inflammatory Disease Activity

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Keywords: Biomarkers, CDAI, Patient-reported outcomes, Crohn's disease, IBD.

CHAPTER 4

BIOMARKER BASED MODELS

4.1 Introduction

CD is a complex disorder that is thought to arise from an interplay between a patient's genetics and their environment. A diagnosis of CD is made based on several factors including clinical history and physical exam, and endoscopic and histologic findings (Lennard-Jones, 1989; Vermeire, Van Assche, & Rutgeerts, 2006). Given the phenotypic heterogeneity of CD, clinical symptoms vary broadly. Multiple diagnostic tools, including the use of objective measures of intestinal inflammation, must be employed to confirm a diagnosis of IBD and to assess and follow inflammatory disease burden. However, the use of diagnostic tools and scoring systems as endpoints in CD are often inconsistent (Sandborn et al., 2002). Frequently, clinical symptoms do not correlate well with the actual burden of intestinal inflammation in terms of location, extent or severity (D'Haens, Geboes, Ponette, Penninckx, & Rutgeerts, 1997; Jones et al., 2008; Mary & Modigliani, 1989). For that reason, the use of these symptoms to estimate inflammatory activity and to adjust therapy may be inaccurate. The incorporation of more objective measures of disease activity into scoring systems may be important in this population and needs to be explored.

Although assessment of clinical symptoms is important for the management of IBD, patient-derived and clinical assessments of disease activity are subjective. Additionally, they may be influenced by other non-inflammatory features of the disease (i.e. intestinal strictures). To measure the impact of clinical symptoms, clinicians often rely on PRO. A PRO is any report or score supplied by the patient about a health condition or treatment. A PRO offers a unique perspective about the impact of chronic disease since it comes directly from the patient without being analyzed or interpreted (Feagan et al., 2011; Guyatt et al., 1989). In the 1970s, the National Cooperative Crohn's disease Study created a validated index of disease severity to assess clinical response to a treatment regimen (Best et al., 1976). The CDAI, derived through a multivariate regression model of 8-variables, has since been widely used in clinical trials in CD (Sandborn et al., 2002). The scoring components of the CDAI are comprised of symptoms reported by the patient (number of liquid stools, abdominal pain and general well-being), along

with laboratory and physical examination parameters (presence of an abdominal mass). In a fourth category patients report current disease-related complications (arthritis, athralgia, iritis, uveitis, anal fissure/fistula, other fistula, fever>37.8° C, erythema nodosum or pyoderma gangrenosum) and opiate use. Lastly, the score requires the measurement of hematocrit and percent deviation from standard weight. Each component does not contribute equally to overall score. Since the CDAI is a composite score including laboratory parameters, it is not considered a pure PRO. However, a significant, negative correlation has been observed between other PROs and the CDAI (Feagan et al., 2011; Ren, Lai, Chen, Irvine, & Zhou, 2007). The CDAI has been shown to correlate poorly with endoscopic disease activity (Gomes et al., 1986; Jones, Loftus, Panaccione, Chen, Peterson, McConnell, et al., 2008; Kiss et al., 2011; Thia et al., 2008). This is perhaps because several components of the CDAI are not CD specific (Williet, Sandborn, & Peyrin-Biroulet, 2014). Recent work has shown the CDAI to be ineffective at discriminating between irritable bowel syndrome (IBS) and active CD (Lahiff et al., 2013). Also, the CDAI score has been shown to have poor reproducibility, with poor sensitivity to changes over time (de Dombal & Softley, 1987). The increasing prevalence of obesity in CD patients and the inclusion of ideal bodyweight in the CDAI score raises concerns about the score's accuracy in measuring the inflammatory disease burden, among other issues. The Food and Drug Administration (FDA) in the United States and Health Canada are moving away from the use of the CDAI to measures that are exclusively derived from patient self-report (Levesque et al., 2015). The addition of PROs to composite scores may provide a unique and patient specific view of the impact of IBD. This qualitative assessment explores issues that may be important to patients, generating knowledge about individual patient experiences in IBD, that may be overlooked by health care professionals (Dür et al., 2014; Finlay & Ballinger, 2006; Mayberry, Lobo, Ford, & Thomas, 2013). However, during the development and validation of PRO, investigators need to consider the varied disease characteristics of IBD patients (i.e., IBD phenotype, location, extent of bowel involvement). Also, outside of the subjectivity of PRO, this type of assessment may burden the patient (U.S. Department of Health and Human Services FDA Center for Drug Evaluation and Research, U.S. Department of Health and Human Services FDA Center for Biologics Evaluation and Research, & U.S. Department of Health and Human Services FDA Center for Devices and Radiological Health, 2006). In the future, it is likely that a pure PRO will be used as a co-

primary endpoint, along with endoscopy, in clinical trials of investigational agents in IBD (Williet et al., 2014; Yellen, Cella, Webster, Blendowski, & Kaplan, 1997).

At the present time, colonoscopy is considered by many to be the gold standard for detecting and quantifying the degree of intestinal inflammation in IBD. However, colonoscopy itself is a surrogate marker for disease activity as it does not capture the transmural nature of the chronic intestinal inflammation of CD. Nevertheless, it does provide information about the location, extent and severity of inflammation and the development of luminal structural complications. Disease extent and severity of mucosal ulceration are important to know as these have been shown to be independent predictors of long-term disease course. As well, mucosal healing (MH) has been associated with significantly fewer hospitalizations, surgeries and intensive care unit stays in patients receiving anti-tumour necrosis factor alpha (anti-TNF) therapy (Baert et al., 2010; Isaacs, 2010; Rutgeerts et al., 2004). Although most of the evidence related to MH is indirect, endoscopic healing of ulcerations has been proposed as a fundamental goal of therapeutic treatment in CD. However, the frequent use of endoscopy to monitor IBD disease activity is burdensome to the health care system and to the patient and may lead to overutilization of scarce endoscopic resources and increased health-care costs. Access to endoscopic resources is also limited in Canada. These limitations prevent frequent endoscopic assessment of disease activity (Konikoff & Denson, 2006). There are several scoring instruments used by endoscopists. The Crohn's disease Endoscopic Index of Severity (CDEIS) is a prospectively developed and validated tool used for the assessment of severity of mucosal inflammation in CD (Mary & Modigliani, 1989). The CDEIS is complex and cumbersome for use in routine clinical practice. A more simplified version of this score, called the Simple Endoscopic Score for Crohn's disease (SES-CD), has been developed and validated (Daperno et al., 2004). The SES-CD score has been demonstrated to be highly correlated with the CDEIS, is much easier to perform and therefore feasible for use in clinical practice (Schoepfer et al., 2008). However, measuring endoscopic response can be difficult without a clear consensus on the SES-CD or CDEIS definition for endoscopic remission or response (Björkesten et al., 2012; Mary & Modigliani, 1989; Schoepfer et al., 2010; Sipponen et al., 2008). On the other hand, the CDAI recognizes clinical remission as a score less than 150, and clinical response, as a decrease of 100 in score from baseline (Vermeire, Schreiber, Sandborn, Dubois, & Rutgeerts, 2010). In our

study, the SES-CD was used as the independent variable, and gold standard measure of intestinal inflammation, against that model variables were compared.

Advances in disease assessment and monitoring have led to the development of non-invasive, objective biomarkers of inflammatory disease activity. A biological marker ("biomarker") is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working Group, 2001). Numerous biomarkers have been evaluated as potential surrogate markers of intestinal inflammation. However, FC, IL-6, and CRP have the most promise, with FC demonstrating the highest sensitivity, specificity and positive and negative predictive values (Andre et al., 1981; Boirivant et al., 1988; Brignola et al., 1986; Fagan et al., 1982; Niederau et al., 1997; Poullis et al., 2002; Røseth et al., 1992; Schoepfer et al., 2008; Sugi et al., 1996; Teahon et al., 1997; Van Assche et al., 2010; van der Sluys Veer et al., 1999; Vermeire et al., 2004; von Roon et al., 2007; Walker et al., 2004). IL-6 and CRP are acute phase proteins that play a vital role in the inflammatory response process (Vermeire et al., 2004). IL-6 is a pro-inflammatory cytokine released by T cells and macrophages originating at the site of inflammation that stimulates hepatocytes to produce CRP. IL-6 is elevated in patients with CD, correlates to clinical disease activity, and is predictive of relapse (Mahida, Kurlac, Gallagher, & Hawkey, 1991; Mitsuyama et al., 1991; Tilg, Dinarello, & Mier, 1997). CRP is produced almost exclusively by hepatocytes in the liver, although extra-hepatic production has been demonstrated (Henriksen et al., 2008). Following an acute phase reaction CRP is rapidly produced, with potential for peak levels reaching between 350-400 mg/L. Normal CRP values fall within 0 – 7.0 mg/L. Serum levels of CRP during the inflammatory process, when an individual is capable of mounting a CRP response, are useful for assessing a patient's risk of relapse and response to medication (Van Assche et al., 2010; Vermeire et al., 2004). Recently, the assessment of hsCRP, that can detect lower concentrations of serum CRP, suggest a stronger association with intestinal disease activity. Ultimately, the specificity of CRP limits its use as a biomarker in CD.

An ideal biomarker would be specific for gastrointestinal inflammation with the potential to supplant endoscopic evaluation in defined circumstances in CD (i.e. monitoring response to

therapy and prediction of disease flare). The inflamed intestinal mucosa is populated with a large number of neutrophils that are in direct contact with the fecal stream. Neutrophil derived proteins, such as FC and lactoferrin, are specific to intestinal inflammation (Abraham & Kane, 2012; Kane et al., 2003; Røseth et al., 2004, 1997, 1992; Tibble et al., 2000; Tibble & Bjarnason, 2001). FC, a calcium-binding protein, is found in neutrophils, and to a lesser degree in monocytes and macrophages, and comprises a large portion of cytosolic (60%) and total (~5%) protein of these cells (Dale et al., 1985; Fagerhol et al., 1990; Røseth et al., 1992). FC plays a regulatory role in the inflammatory process and has bacteriostatic and fungistatic properties. Increased levels of FC have been consistently observed in IBD, colorectal carcinoma, and nonsteroidal enteropathy patients (Kristinsson et al., 1998; Meling et al., 1996; Røseth et al., 1997, 1992; Teahon et al., 1997; Tibble et al., 2001; Tibble et al., 1999). FC values in the non-inflamed intestine have been validated to fall below 50 µg/g. FC has been shown to be superior to serological markers (i.e. CRP) in its ability to diagnose IBD (von Roon et al., 2007). A meta-analysis evaluating the operating characteristics of FC in diagnosing IBD demonstrated pooled sensitivity and specificities of 93% and 96%, respectively (van Rheenen et al., 2010). FC also correlates positively with endoscopic activity (i.e. SES-CD), with correlation coefficients ranging from 0.48–0.73, sensitivities ranging from 81%–91%, and specificities from 58%–100% (D'Incà et al., 2007; Schoepfer et al., 2010; Sipponen et al., 2010). In a recent study, FC not only demonstrated a strong relationship with endoscopic disease, but was observed to predict the presence of large ulcers with a sensitivity of 60.4% and a specificity of 79.5% (250 µg/g cut-off value) (D'Haens et al., 2012). Additionally, normal FC concentrations have been shown to be a consistent surrogate markers for endoscopically and histologically inactive disease (Jones, Loftus, Panaccione, Chen, Peterson, McConnell, et al., 2008; Sipponen et al., 2010; Sipponen, Kärkkäinen, et al., 2008). The measurement of fecal biomarkers, in combination with other factors that influence inflammation, may aid in the development of non-invasive indices of disease activity.

Many patient-specific and environmental factors impact disease activity in CD. Although not included in existing indices, the impact of these factors on endoscopic disease activity warrants further investigation. Cigarette smoking has been shown to be an independent risk factor for clinical, surgical and endoscopic recurrence in CD, with further influence on disease

activity after surgery (Loftus, n.d.; Somerville et al., 1984; Sutherland, Ramcharan, Bryant, & Fick, 1990; Tobin, Logan, Langman, McConnell, & Gilmore, 1987). Other anthropometric measures, particularly body composition, could influence disease activity. Alterations to body composition could impact disease course and response to therapy in CD (Bryant, Trott, Bartholomeusz, & Andrews, 2013). Recently, obesity in CD patients has been associated with a more severe disease course as well as higher concentrations of inflammatory biomarkers (Fink, Karagiannides, Bakirtzi, & Pothoulakis, 2012; Hass, Brensinger, Lewis, & Lichtenstein, 2006). The accumulation of mesenteric white adipose tissue (WAT), central in CD and obesity, will increase the secretion of cytokines from adipose tissue ("adipokines"). Alterations to levels of circulating adipokines may impact the pathogenesis and disease course of IBD (Karmiris, Koutroubakis, & Kouroumalis, 2008). The rate of surgical resection, a common occurrence in the first three years of CD, may offer prognostic value (i.e. patients that underwent surgery had more severe disease) in stratifying disease activity (Sands et al., 2003). Dietary factors may also impact disease activity. Therefore, smoking status, surgical resection and BMI were assessed in our study.

4.2 Objectives

The aim of this study was to determine the relationship between individual components of the CDAI (using both PRO- and biomarkers variables) and other disease activity variables and the SES-CD, in the effort to create a sensitive and specific model (including a PRO-exclusive model) to predict endoscopic disease activity.

4.3 Materials and Methods

This was a cross-sectional observational study conducted between 2004 and 2006. One hundred and sixty-four consecutive adults with an established diagnosis of Crohn's disease, undergoing clinically indicated ileocolonoscopy were recruited to participate in this study (Jones, Loftus, Panaccione, Chen, Peterson, McConnell, et al., 2008). Recruitment was held at two tertiary care centers, the University of Calgary, Calgary, AB, Canada and the Mayo Clinic College of Medicine, Rochester, MN, USA. Patients were excluded if they had an alternate condition that could elevate CRP or FC concentrations including associated autoimmune diseases, active infection, cancer, ileostomy, or evidence of a small bowel obstruction. Clinical, endoscopic, serologic and fecal inflammatory biomarker measurements were collected prospectively. Data collection was performed through performance of patient interviews, colonoscopy, and laboratory measures. CDAI was used to determine clinical disease activity that relies on a 7-day patient recall (Frenz, Dunckley, Camporota, Jewell, & Travis, 2005). Patients with a CDAI score > 150 points were considered to have active disease. Those with CDAI score < 150 were considered to be in clinical remission. Since the CDAI uses ideal body weight as an indication of changes in body composition, scores for this variable can be both positive and negative (and therefore add to or subtract from the total score). The conventional practice when calculating the CDAI is to truncate negative scores at 10. The variables used to diagnose and assess disease activity in CD are summarized in **Table 4.1**.

Table 4.1 Variables potentially predictive of disease activity in CD

Patient reported outcome (PRO)	**Clinical**	**Laboratory**	**Endoscopic**
Number of liquid stools	*Disease Complications*	*Hematocrit*	Presence and size of ulcers^
Abdominal pain	*Ideal Body Weight*	hsCRP	Extent of ulcerated surface^
General well-being	*Opiate use*	FC	Extent of affected surface^
	Abdominal mass	IL-6	Presence and type of narrowing^
	BMI	Histology	
	Smoking status	Lf	
	Surgical resection		

BMI, body mass index, **hsCRP**, high sensitivity C - reactive protein, **FC,** fecal calprotectin, **IL-6,** interleukin-6, **Lf**, lactoferrin, ***Hematocrit***, italics denotes CDAI parameter, ^ denotes simple endoscopic score – Crohn's Disease (SES-CD) parameter.

Endoscopic disease activity was scored by colonoscopists who were blinded to the laboratory and CDAI results using the SES-CD. A SES-CD score of > 7 points was indicative of active disease. Serum concentrations of hsCRP were measured on a Hitachi 912 (Roche Diagnostics Corp, Indianapolis, IN) automated chemistry analyzer using a high-sensitivity polystyrene particle–enhanced immunoturbidimetric assay from DiaSorin (Stillwater, MN). Serum concentrations of IL-6 were measured by quantitative, 2-site sandwich enzyme immunoassay (R&D Systems, Minneapolis, MN). Fecal concentrations of FC were measured by a quantitative enzyme immunoassay using polyclonal rabbit antibodies (*Phi-Cal*; Genova Diagnostics, Asheville, NC). Fecal concentrations of Lf were measured by a quantitative enzyme immunoassay (*IBD-SCAN*, TechLab, Inc, Blacksburg, VA). Specific information on the analytical performance of these laboratory tests, along with characteristics of these biomarkers, can be found in the original article (Jones, Loftus, Panaccione, Chen, Peterson, McConnell, et al., 2008).

In our study, the SES-CD score was the independent variable. Our analysis included the eight original CDAI variables along with six new variables: BMI, smoking status, surgical resection, FC, IL-6, and hsCRP (**Table 4.2**). Since biomarkers are not observed as normally distributed but rather with skewed distribution they were log-transformed. These variables were analyzed, in univariate analysis, and then in multivariate models for the prediction of SES-CD. Simple Poisson regression was performed on each of the variables to identify those that may have an effect on the SES-CD. From this analysis, variables that correlated well with the SES-CD were included in our new model. Akaike information criterion (AIC) values were used to compare similar variables (i.e. BMI and ideal body weight). To create a new predictive model, a robust, multivariate Poisson regression model, named **PRO+**, was built using a cross-validated bootstrapping approach (bagging).

Table 4.2 Variables found in study models

CDAI	PRO+	PRO-exclusive
Number of liquid stools	Number of liquid stools	Number of liquid stools
Abdominal pain	Abdominal pain	Abdominal pain
General well-being	General well-being	General well-being
Symptom Categories	Symptom Categories	Symptom Categories
Opiate use	Hematocrit	**BMI**
Abdominal mass	**hsCRP**	
Hematocrit	**FC**	
Ideal Body Weight	**BMI**	

BMI, body mass index, **hsCRP**, high sensitivity C - reactive protein, **FC,** fecal calprotectin. Non-CDAI variables included in the model are in **bold**.

To evaluate the operating characteristics of PRO+, a receiver operating characteristic (ROC) curve was used to evaluate the prediction of active disease using SES-CD at two levels (SES-CD of ≥7 versus ≤ 6, for moderate disease SES-CD >1). The ROC curve provided sensitivity, specificity and area under the curve (AUC) values for our CDAI, FC, PRO+ and pro-exclusive models.

4.4 Results

One hundred and eighty patients were screened for potential enrolment and 164 were subsequently enrolled (see Section **4.3** for inclusion/exclusion criteria). The demographic characteristics of the patients are shown in **Table 4.3**. The mean age of the patients was 41 years (range 18 - 75) and the mean duration of disease was 12 years (range 0.5 - 40). Notably, 23% of patients were receiving medications that have been shown previously to decrease CRP concentration including statins and nonsteroidal anti-inflammatory drugs. Forty-four percent of patients had ileocolonic disease, with 32 and 24% being restricted to the ileum and colon, respectively. Sixty percent of patients had inflammatory (nonstricturing, nonpenetrating) disease, while 35% and 5% had stricturing and penetrating disease, respectively. Seventeen percent of patients were receiving anti-TNF therapy with infliximab or adalimumab and 30% of patients were receiving corticosteroids.

Table 4.3 Clinical and Demographic Characteristics (n=164)

	Frequency	(%)
Sex		
Male	68	**40**
Female	97	**59**
Smoking Status		
Smoker	26	**16**
Non-smoker	134	**82**
Missing	4	**2**
Medications		
Anti-TNF	29	**17**
Steroids	51	**30**
Immunosuppressants	81	**49**
NSAIDs	20	**20**
Statins	5	**3**
Montreal		
Ileal	53	**32**
Ileocolonic	72	**44**
Colonic	40	**24**
Stricturing	57	**35**
Penetrating	8	**5**
Nonstricturing, nonpenetrating	99	**60**
Age of diagnosis (y)		
< 16	23	**14**
17 - 40	114	**69**
> 40	29	**17**

NSAIDs, non-steroidal anti-inflammatory drugs, **Anti-TNF**, anti-tumour necrosis factor.

A poor relationship was observed between CDAI and SES-CD (**Figure 4.1**). Univariate pre-screening revealed that antidiarrheal/opiate use, smoking status and presence of resection correlated poorly with the SES-CD score and were dropped from the model (**Table 4.4**). Additionally, abdominal mass (a localized enlargement or swelling found in the abdomen) was not present in any of the subjects and was dropped from the model. Both hsCRP and IL-6 were found to correlate well with SES-CD, but their collinearity precluded including both in the model. As hsCRP is more clinically accessible and has a slightly stronger relationship with SES-CD, IL-6 was excluded from the multivariate model. BMI and ideal bodyweight had similar AIC values, but again due to collinearity could not both included in the model. BMI was selected for the model since it has been more recently used in work exploring associations between obesity

and disease activity in IBD. Neither variable contributed significantly to the final prediction in the multivariate model.

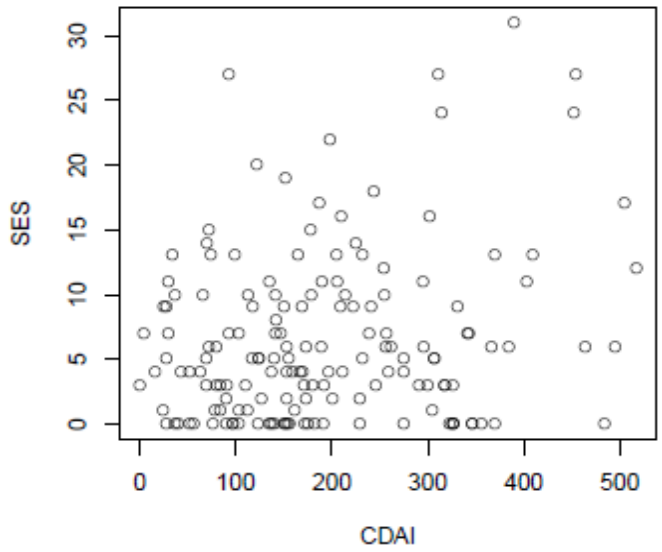

Figure 4.1 Relationships between CDAI and SES-CD. Plot showing weak relationship between the Crohn's disease Activity Index (CDAI), a tool typically used in clinical trials, with the gold standard for assessing disease activity in Crohn's disease (CD), the Simple Endoscopic Score for Crohn's disease (SES-CD).

Table 4.4 Results of univariate Poisson modelling (n=164)

	p value	**AIC**
Existing Variables		
(1) number of liquid stools	<0.0001	1401.6
(2) abdominal pain	0.0015	1447.5
(3) general well-being	<0.0001	1432.7
(4) symptom categories	<0.0001	1390.6
(5) lomotil/opiate use	0.9067	1444.5
(6) abdominal mass	0.0019	1434.9
(7) hematocrit	<0.0001	1408.6
(8) % from standard weight	0.0013	1448.8
New Variables		
BMI	<0.0001	1351.7
Smoker	0.0794	1462.3
abdominal resection	0.8763	1455.8
(log) FC	<0.0001	1020.6
(log) IL-6	<0.0001	1161.0
(log) hsCRP	<0.0001	1158.1

BMI, body mass index, **(log),** logarithmic scale, **FC**, fecal calprotectin, **IL-6**, interleukin-6, **hsCRP**, high sensitivity C-reactive protein, **AIC**, Akaike information criterion.

The remaining variables were used to model PRO+ (**Table 4.5**). Three variables were significantly and consistently correlated to SES-CD in the multivariate Poisson regression model: number of liquid or soft stools, FC and hsCRP**.**

Table 4.5 Summary of Multivariate Poisson Regression Bootstrapping

PRO-exclusive	**median p value**	PRO+	**median p value**
Existing Variables		**Existing Variables**	
(1) number of liquid stools	0.0018	(1) number of liquid stools	<0.0001
(2) abdominal pain	0.0846	(2) abdominal pain	0.0520
(3) general well-being	0.0053	(3) general well-	0.2404
(4) symptom categories	0.0001	(4) symptom categories	0.2283
(7) hematocrit	0.0024	(7) hematocrit	0.1261
New Variables		**New Variables**	
BMI	0.0147	BMI	0.2103
		(log) FC	<0.0001
		(log) hsCRP	<0.0001

BMI, body mass index, **(log),** logarithmic scale, **IL-6**, interleukin-6, **hsCRP**, high sensitivity C-reactive protein.

For the prediction of endoscopic disease activity (SES-CD ≥7 versus ≤ 6), the AUC for PRO+ was 0.81 AUC (95% CI: [0.802 0.817]) with sensitivity and specificity of 66.0% and 87.2%. In comparison, the CDAI AUC was 0.54 (95% CI [0.534, 0.552]) with sensitivity and specificity of 58.8% and 58.9%, the PRO-exclusive was 0.62 AUC (95% CI: [0.609, 0.628]) with sensitivity and specificity values of 62.7% and 60.6%, and FC was 0.74 AUC (95% CI: [0.732, 0.749]) with sensitivity and specificity values of 76.5% and 45.2%. The ROC used in predicting active disease between these four models is shown in **Figure 4.2**.

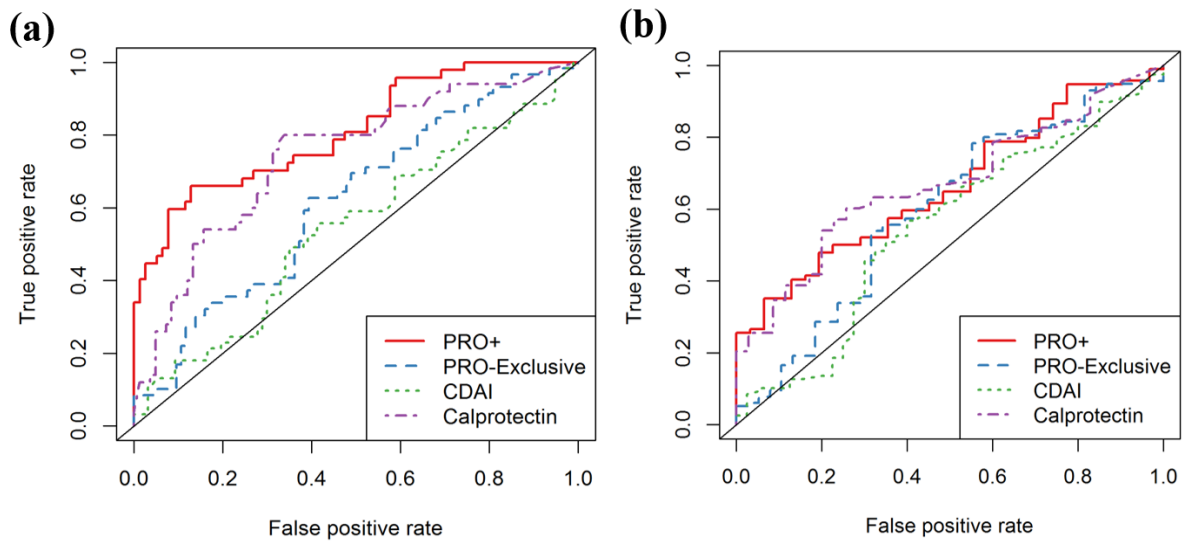

Figure 4.2 Receiver Operating Curve (ROC) for predicting SES-CD in CD. Calprotectin (FC), PRO+ and pro-exclusive models **(a)** SES-CD of ≥7 versus ≤ 6. PRO+: 0.81 AUC, 66.0% sensitivity, 87.2% specificity, PRO-exclusive: 0.62 AUC, of 62.7% sensitivity, 60.6% specificity, CDAI: 0.54, 58.8% sensitivity, 58.9% specificity, FC: 0.74 AUC, 76.5% sensitivity, 45.2% specificity. **(b)** For moderate disease SES-CD ≥1 versus ≥2. PRO+: 0.66 AUC, 35.1% sensitivity, 93.5%, PRO-exclusive: 0.60 AUC, of 78.3% sensitivity, 44.7% specificity, CDAI: 0.54, 56.8% sensitivity, 60.0% specificity, FC: 0.67 AUC, 60.2% sensitivity, 74.3% specificity.

4.5 Discussion

Our understanding of what drives clinical symptoms in patients with IBD has evolved. The recognition that non-inflammatory mechanisms can cause symptoms that may resemble those experienced by patients with active intestinal inflammation has led to an agnostic re-evaluation of commonly used measurement tools of CD disease activity. Inconsistencies observed in the associations between clinical symptoms and endoscopic disease severity in CD are well documented (Jones et al., 2008). Despite this, the CDAI, a composite disease activity measure, is the most commonly used scoring tool for assessing disease activity in clinical trials (Sandborn et al., 2002). This study confirmed the lack of accuracy of the CDAI for assessment of endoscopic disease activity and evaluated the ability of a new model to predict endoscopic inflammatory disease activity. The weak correlation observed between the CDAI and SES-CD scores (**Figure 4.1**) may exist for many reasons. The CDAI includes the use of different disease-related symptoms, some of that are less specific for intestinal inflammation by virtue of the fact that they are more subjective in nature. Additionally, the CDAI includes variables that are not

applicable to a number of subgroups of CD patients. For example, CD patients in objective remission (clinically and endoscopically) with IBS symptoms have a greater likelihood of being misclassified as having active CD given symptomatic overlap between selected subtypes of IBS and IBD (i.e. diarrhea and abdominal pain). Likewise, the CDAI includes other subjective, non-specific symptoms that may be caused by systemic factors or diagnoses that are unrelated to IBD. For example "general well-being", included in the CDAI could be influenced by several factors that are not IBD related. These factors limit the utility and accuracy of the CDAI, and although validated, make it an unreliable surrogate measure of endoscopic disease activity (**Figure 4.2**).

Inclusion of biomarkers, such as FC and hsCRP, into disease activity scores improves the prediction of endoscopic disease activity (**Figure 4.2**) (Björkesten et al., 2012; Langhorst et al., 2008; Schoepfer et al., 2010). However, the published literature pertaining to the creation of non-invasive composite scoring models is limited. Similar to our study, Khanna et al. (2015) showed the use of 2- or 3-item PRO derived from CDAI diaries (stool frequency, abdominal pain, and general well-being) were suitable to predict CDAI-defined outcomes and responsiveness to medical therapy. Bjorkesten et al. (2012) observed that FC alone was effective in predicting endoscopic remission with sensitivity and specificity values of 84% and 74%, respectively. They also showed that FC combined with the HBI was superior to FC alone as a surrogate measure of endoscopic disease activity. However these observations did not reach statistical significance. Langhorst et al. (2008) observed the performance of the CDAI, CRP and three fecal biomarkers (FC, lactoferrin and polymorphonuclear neutrophil elastase concentrations) in distinguishing active and inactive CD. Their analysis showed no singular fecal biomarkers was consistently reflective of endoscopic inflammation and that they all performed similarly in terms of their operating characteristics (AUC, sensitivity and specificity for lactoferrin was 0.87, 85% and 77.2%, for FC 0.89, 100% and 36.7%, and 0.75, 60.7% and 75.9%, for CRP). However, all fecal biomarkers were superior to serum CRP concentration in their prediction of endoscopic disease activity. Also, this group created a categorical (positive or negative) comprehensive index, that included three stool biomarkers (marked positive if two of three stool parameters were above respective cut-offs), CRP, and the disease-specific activity index (CAI for UC and CDAI for CD) (Langhorst et al., 2008). This index was rated positive if two of three categories were observed

positive and expressed as a percentage correspondence for active and inactive disease status. This index had a 76.7 and 95.3% diagnostic accuracy in CD and UC patients, respectively (Langhorst et al., 2008). This composite scoring index outperformed both serum CRP concentration and the CDAI, but not FC concentration for estimation of endoscopic disease activity in CD. This observation may be reflective of the fact that CD is a very heterogeneous disease comprised of differences in disease location, extent and phenotype, all of which alter the observed concentration of FC. Unlike CD, UC is a much more homogeneous disease entity characterised by universal rectal involvement with a continuous pattern of inflammation of various extent. There is further evidence to suggest that FC concentration is dependent on the location of inflammation within the intestine (af Björkesten et al., 2012; Schoepfer et al., 2010). Schoepfer et al. (2010) analyzed the relationship between FC and disease location (ileum, colon, and ileocolonic) in CD, observing the strongest correlation between FC and SES-CD in ileocolonic disease ($r=0.795$, $p<0.001$). This is direct evidence that extent and location of inflammatory disease influences FC concentration. Additional studies should stratify subjects by subgroups of CD based on disease location (colonic vs. ileocolonic), disease extent and disease behaviour (inflammatory vs. stricturing vs. penetrating). Such stratification would allow the development and validation of such models specific to CD subgroups and generation of results that will be more applicable to a heterogeneous patient population.

The PRO+ model out-performed both the CDAI and PRO-exclusive model in predicting the SES-CD in a heterogeneous sample of CD patients. Although an improvement over the existing CDAI, the new model could still be more accurate. Additionally of note, the use of only FC to predict SES-CD was more accurate than the PRO-exclusive model. A full calculator can be found in **Figure 4.3**. Previous studies have shown better operating characteristics with fecal biomarkers alone, although none of these studies attempted to include PRO (D'Incà et al., 2007; Schoepfer et al., 2010; Sipponen et al., 2010). The inclusion of PRO may address more global, patient specific aspects of disease activity that biomarkers fail to capture, leading to a more accurate model. Additionally, the PRO-exclusive model, including number of liquid stools and abdominal pain, still out performed the CDAI. This observation draws attention to parameters of the CDAI that may no longer be useful, and could actually detract from the score's accuracy.

This observation raises questions about the ongoing utility and applicability of the CDAI for disease assessment in CD.

In contrast to previously performed studies evaluating the associations between inflammatory biomarkers and endoscopic disease activity, we included a larger number of clinical variables in addition to biomarkers of inflammation (Denis, Reenaers, Fontaine, Belaïche, & Louis, 2007; Solem et al., 2005). Modest correlations between the SES-CD and serological and fecal biomarkers have been observed in previous studies, suggesting that one biomarker alone may not be reliable as a surrogate biomarker of endoscopic disease activity. This highlights issues related to both inter and intra-individual biomarker concentration variability in CD populations. Therefore, the inclusion of additional validated variables were a methodological strength in our study, since we could evaluate each variable individually, and then as part of remodelled score.

The results of the PRO+ model highlight the importance of including variables that are both objective and patient-derived in nature. The combination of these variables appears to out-perform traditional validated measures as well as measures using solely objective biomarkers of intestinal inflammation. Of the five variables included, three (number of liquid stools, abdominal pain, and symptom/complication categories) were included in the original CDAI tool. Number of liquid stools and presence and severity of abdominal pain are considered PRO-variables. The other two variables, hsCRP and FC, are biomarkers, surrogate markers of inflammation. FC, a neutrophil derived inflammatory marker, has been shown perhaps to be the most sensitive and specific surrogate marker of mucosal inflammation (D'Haens et al., 2012). Our study confirms the import relationship between fecal biomarkers and the presence of active endoscopic disease.

Interestingly, variables hypothesized to impact endoscopic disease activity, such as smoking status, body composition and surgical resection, did not contribute and were dropped from the model. Some of the CDAI variables, such as presence of an abdominal mass, weren't present in this study population. Since, our study was limited in size (n=164), future validation studies should confirm this relationship. Additionally, although the use of BMI did not improve the prediction of endoscopically active disease, this may be due the inaccuracy of BMI as a

marker of body fat composition, and should not dismiss the relationship between white adipose tissue (WAT) and disease activity. Future research should include more accurate, prospectively collected measurements of body composition and fat mass, such as dual-energy x-ray absorptiometry.

The new model has limitations. With the inclusion of biomarker concentrations, the new model still requires the collection and testing of fecal and serum samples. Thus, the avoidance of diagnostic testing is not averted as it would be with a PRO. However, the inclusion of biomarkers also provides an accurate surrogate measure of disease activity, which is less costly and invasive than colonoscopy. The use of this type of model has far reaching implications; an accurate scoring index would limit the need for colonoscopy in clinical practice, therefore, reducing the burden on the health care related to monitoring of CD disease activity and provide less expensive and more accessible primary outcomes for clinical trials.

4.6 Conclusion

Biomarker enhanced CD disease activity models provide clinicians with a relatively non-invasive, and inexpensive surrogate measure of endoscopic disease activity, with greater predictive accuracy. These attributes give biomarkers ideal clinical utility. The inclusion of biomarkers in scoring models could influence patients that are selected for clinical trials, and could improve the ability to predict disease relapses and response to therapy in clinical practice. Some PRO still possess clinical utility and their use should be continued. . Our study showed the poor relationship between the CDAI and the SES-CD. Incorporation of inflammatory biomarkers into models improved accuracy for endoscopically active disease.

The long PRO+ model can be calculated as follows:

USASK *= e^[0.473 -0.002(BMI) + 0.214 (FC) + 0.195 (hsCRP) + 0.007 (# of liquid stools) -0.016(abdominal pain) + 0.004 (general well being) + 0.054 (symptom/complications) - 0.003(HCT)]*

The simplified model, PRO+, can be calculated as follows:

USASK *= e^[0.473 + 0.214 (FC) + 0.195 (hsCRP) + 0.007 (# of liquid stools)) + 0.004 (general well being) + 0.054 (symptom/complications)]*

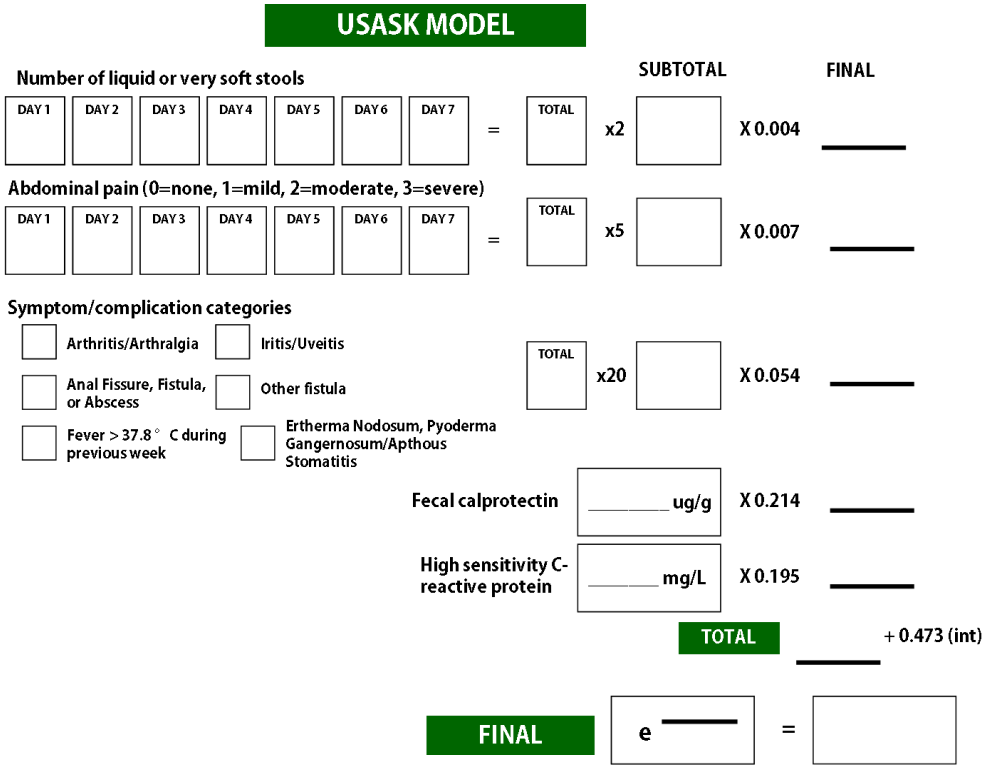

Figure 4.3 PRO+ full scoring model

CHAPTER 5

BIOMARKERS ARE PREDICTIVE OF DISEASE ACTIVITY AND ASSOCIATED WITH MEASURES OF CARBOHYDRATE QUALITY IN NEWLY DIAGNOSED IBD PATIENTS

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Not yet published

Clinicians in IBD apply a combination of symptom scoring, clinical examination, laboratory indices, and radiology, to the “gold-standard” of endoscopy with biopsy, to make key decisions, such as diagnosis, assessment of disease severity, medical therapy and outcome. The use of biological markers ("biomarker"), objective characteristics of normal or pathogenic processes, could improve decision making at these times, particularly in lieu of endoscopic information. However, previous IBD cohorts exploring predictors of disease activity and course have been limited by heterogeneity and confounding effects within the sample population. This chapter focuses on a newly diagnosed cohort of IBD patients, which may remove some of the limitations of previous cohort studies, including disease onset and patient heterogeneity. Additionally, though frequently implicated in pathogenesis and treatment, there is limited evidence regarding the influence of diet and nutritional interventions in IBD. Observing nutritional trends in the period around diagnosis may shed light in this area. To the best of our knowledge, no nutritional assessments have been conducted in newly diagnosed IBD patients in Canada. Observation of nutritional trends, in addition to the novel study of fecal biomarkers in this cohort, makes this study distinctive.

Author role: I completed the lab analysis, statistical analysis and manuscript preparation and writing for this chapter.

Biomarkers are predictive of disease activity and associated with measures of carbohydrate quality in newly diagnosed IBD patients

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Keywords: Inflammatory Bowel Disease, Biomarkers, Calprotectin, Lactoferrin.

5.1 Introduction

IBD encompasses a multisystem group of disorders, with underlying chronic inflammation and specific clinical and pathological features, that primarily affects the GI tract (Burri & Beglinger, 2012). Classical IBD consists of two main forms: CD and UC. Although generally grouped together, CD and UC differ with respect to some important clinical, immunophenotypic and pathologic characteristics, including disease location, complications and select histopathological features (Bamias et al., 2005; Podolsky, 2002). Chronic inflammation leads to IBD related symptoms. Therefore, clinical assessment of inflammation is key to the diagnosis and monitoring of IBD (Konikoff & Denson, 2006). Since inflammation may be subclinical, and thus difficult to observe and assess by patients or physicians, various techniques and systems have been developed to quantify the severity and extent of this inflammation (Konikoff & Denson, 2006). Clinicians apply a combination of symptom scoring, clinical examination, laboratory indices, and radiology, in addition to the current gold- standard of endoscopy with biopsy to make a diagnosis, assess severity and predict the outcome of disease (Vermeire et al., 2006).

The clinical symptoms of IBD, particularly CD, are not specific and unfortunately no hallmark sign or symptom exists (Burri & Beglinger, 2012). There is considerable overlap in the symptoms found in functional disorders, such as IBS and organic diseases like IBD. Thus, differentiating between the organic and functional disease is difficult and often requires a colonoscopy with histology. Laboratory markers that are specific to IBD and could accurately detect inflammation and monitor disease would be useful clinically (Abraham & Kane, 2012). A biological marker ("biomarker") is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working Group., 2001). A biomarker, or set or markers, to fulfill this role would provide an objective measure of inflammation and disease activity while avoiding invasive and expensive procedures (Vermeire et al., 2006).

CRP is a vital acute phase proteins in humans (Vermeire et al., 2004). Following an acute phase reaction, such as gut inflammation, CRP is rapidly produced, with potential for peak levels reaching between 350-400 mg/L. However, since it may be affected by other non-IBD related

phenomena, the utility of CRP in IBD is limited. Historically, a notable difference exists between the strong CRP responses in CD compared with the moderate, weak or absent CRP responses in UC, despite active inflammation (Vermeire et al., 2006). hsCRP was observed to be more effective (100% sensitivity/67% specificity) in differentiating between functional and new IBD diagnoses (Poullis et al., 2002). Unfortunately, the correlation between CRP and disease activity in IBD has been inconsistent (Andre et al., 1981; Boirivant et al., 1988; Brignola et al., 1986; Buckell et al., 1979; Fagan et al., 1982; Niederau et al., 1997). The more recent assessment of hsCRP in CD patients, though, suggests a stronger association with disease activity. Additionally, serum levels of CRP (when an individual mounts a CRP response) are useful for assessing a patient's risk of relapse and response to medication (Van Assche et al., 2010; Vermeire et al., 2004).

Although serum markers are useful in assessing active inflammation, they are non-specific and elevated in conditions unrelated to IBD. The inflamed intestinal mucosa is populated with a larger number of neutrophils that are in direct contact with the fecal stream. Neutrophil derived proteins, calprotectin and lactoferrin being the most widely studied, present the most ideal type of biomarkers in the study of gut inflammation (Abraham & Kane, 2012). Concentration of FC is consistently increased in IBD, colorectal carcinoma, and nonsteroidal enteropathy patients (Kristinsson et al., 1998; Meling et al., 1996; Røseth et al., 1997, 1992; Teahon et al., 1997; Tibble et al., 2001; Tibble et al., 1999). Similar to FC, lactoferrin has been observed to be very accurate in diagnosing IBD. In discriminating from IBS, Schoepfer and colleagues (2008) found that lactoferrin was 91% accurate. Overall accuracy for discrimination of IBS from patients with CD in remission (CDAI<150) was 90% for both lactoferrin and FC (Schoepfer et al., 2008). A meta-analysis conducted by von Roon and colleagues (2007) found FC to be superior to serological markers (i.e. CRP, ESR) in its ability to diagnose IBD (von Roon et al., 2007). FC displays modest to good correlation with endoscopic activity (Burri & Beglinger, 2012). Additionally, levels of fecal lactoferrin are increased in active UC and CD. Lactoferrin is also elevated in inactive IBD, above levels from IBS patients and healthy controls. Furthermore, fecal lactoferrin concentration has a 93% correlation with measures of disease activity (Sugi et al., 1996; van der Sluys Veer et al., 1999; Walker et al., 2004). The use of fecal

biomarkers as surrogate markers of inflammation and their clinical utility holds promise, thus, warrants further investigation.

The study of nutrition and IBD can be divided into three main categories: specific dietary components in pathogenesis, malnutrition or nutritional deficiencies in disease course, and nutritional therapy in response to the disease (Geerling, Stockbrügger, et al., 1999). Nutritional deficiency in IBD is well documented, largely due to the use of objective markers of nutritional status (micronutrients in bloodstream etc.) in a relatively homogeneous patient population (active disease, inpatients) (Geerling et al., 2000; Han et al., 1999; Vagianos et al., 2007). However, the influence of diet in the pathogenesis and treatment of IBD is not well known. Studying newly diagnosed patients, whilst collecting endoscopic and biological markers of inflammation, may remove some of the limitations of previous cohort studies, including disease onset and patient heterogeneity.

Dietary carbohydrates, the principal dietary constituents that control the rise in blood glucose (postprandial glycemia) and insulin secretion after a meal, have been long implicated in the development of IBD (Brand-Miller, 2004). In observational studies, an increase in dietary sugar consumption has been consistently reported in IBD. (Geerling, Stockbrügger, et al., 1999; Järnerot, Järnmark, & Nilsson, 1983; Kasper & Sommer, 1979; Martini & Brandes, 1976; Mayberry et al., 1981; Mayberry, Rhodes, & Newcombe, 1978, 1980; Miller et al., 1976; Penny et al., 1983; Rawcliffe & Truelove, 1978; Silkoff et al., 1980; Thornton, Emmett, & Heaton, 1979). However, clinical trials testing the therapeutic effect of a low-refined carbohydrate, high-fibre diet in the treatment of IBD produced conflicting results (Jones et al., 1985; Lorenz-Meyer et al., 1996; Ritchie et al., 1987). Recently, the consumption of wheat-bran had no adverse effects in CD, while increasing QOL and GI function (Brotherton, Taylor, Bourguignon, & Anderson, 2014). However, a systematic review in the area showed limited weak evidence for the effectiveness of fibre in improving disease outcomes (Wedlake, Slack, Andreyev, & Whelan, 2014). The GI is used to quantify the glycemic response to carbohydrate-containing foods (Jenkins et al., 1981). The GL of a food is the product of the GI and the amount of carbohydrate in the food. In recent years, the values of GI and GL in the human diet have increased, mainly due to increases in carbohydrate consumption and advances in food processing (Ludwig, 2002). Excess postprandial blood glucose has been implicated as a developmental risk factor for

cardiovascular disease and diabetes in prospective observational studies, but has never been studied in IBD (Beulens et al., 2007; Schulze et al., 2004). To the best of our knowledge, no nutritional data in newly diagnosed Canadian IBD patients exists.

5.2 Objectives

Previous cohort studies in IBD exploring predictors of disease course have had limitations due to the heterogeneity and confounding effect of numerous subject and disease-related characteristics of the subjects within the sample population. When studying a population of patients with long-established disease, patients are subject to different medications, complications and surgeries distorting the usefulness and calculation of biomarker concentrations and measures of disease activity. Thus, studying newly diagnosed patients is important for observing a homogenous sample population; consequently limiting confounders.

Biomarkers, together with endoscopic appearance, have never been evaluated together for their joint predictive ability in disease course. The primary objective of this research is to develop a single-center, prospective cohort of newly-diagnosed adults with IBD, in the effort to evaluate the predictive performance of select endoscopic, serologic, and fecal biomarkers in long-term disease course and response to therapeutics. A secondary objective of this study is to provide a nutritional assessment in newly diagnosed patients, observing nutrient intakes during the onset of IBD

5.3 Materials and methods

Patient recruitment: Eligible subjects were those having a new diagnosis of IBD within the past 12 months by treating physicians at the Royal University Hospital and the Saskatoon area. Individuals ages 18 and older with a diagnosis of IBD based on standard criteria and able to provide written informed consent were eligible to participate in the study conducted from November 2012 to July 2014 (Lennard-Jones, 1989). Exclusion criteria in this cohort consisted of patients with infectious diseases, end-stage renal, cardiovascular or hepatic disease or who are pregnant. This study was approved by the University of Saskatchewan's Biomedical Research Ethics Board (Bio-REB) (Bio# 09-26).

Experimental design: Patient demographics (age, gender, disease type, location, medication therapy, smoking status) and anthropometric characteristics (height, weight, BMI) were collected from all participants. A nutritional assessment was conducted at baseline by a FFQ. At baseline and follow-up (3 – 6 months), detailed endoscopic and clinical disease activity measures were calculated. At baseline and follow-up visits (3 – 6 months depending on disease activity) over a two-year period, blood and fecal samples were collected for biomarker determination.

Blood was drawn by trained Royal University Hospital personnel and used for hsCRP determination. Fecal samples (two 50-gram aliquots) were collected in sterile containers, one left thawed for biomarker determination, and one frozen immediately and delivered to the Mary Irwin Laboratory of Nutrition at the University of Saskatchewan for analysis found in **Chapter 7**.

Disease Activity: Clinical disease activity was scored using the CDAI and HBI for CD and partial Mayo (pMayo) and PT score for UC. CDAI was categorized as active ≥ 150, inactive < 150 and HBI as remission ≤ 2, active 3-7 and severe ≥ 8. Partial Mayo was categorized as inactive ≤2, mild 3-4, moderate 5-6, and severe 7-9. The PT score was only used in the longitudinal analysis and, therefore, not categorized. Endoscopic disease activity was scored by colonoscopists using the SES-CD and Mayo Score for UC. SES-CD was categorized as inactive 0-2, mild 3-6, moderate 7-15, severe ≥ 16 and Mayo score as inactive ≤ 2, mild 3-5, moderate 6-10, severe 11-12.

Biomarker analysis: hsCRP was tested using the high sensitivity CRP (latex) reagent on the c501 analyzer (Roche Diagnostics Canada, Laval, QC) using the manufacturer's reagents and calibrators (Sánchez et al., 2002). Analysis was completed by the Saskatoon Health Region.

FC was determined using a Quantum Blue® FC High Range Rapid Test using the Quantum Blue Reader® point-of-care (POC) desk-top reader (ALPCO Immunoassays, Salem, NH). Approximately 30 mg of fecal sample was pressed into a base cap and fitted on the extraction tube. This device was filled with 4 mL of extraction buffer and homogenized by vortex for 1 minute. A 1:16 dilution was performed with chase buffer and 80 µl was positioned

into the test cartridge for analysis. Each run took 15 minutes to complete and results were displayed in micrograms per gram (µg/g) on the POC display.

Lf was determined using IBD-SCAN® (TechLab, Inc, Blacksburg, VA). Approximately 450 mg of fecal sample was weighed in an Eppendorf tube and diluted at 1:100 to 1:10,000. Test samples, standards and quality controls were plated in a 96-well plate provided with the kit. The plate was read at 450/620 nanometres wavelength with a microplate reader and results were calculated in micrograms per millilitre (µg/mL).

Nutritional Assessment: Dietary information was captured using a FFQ, the 98 Block Questionnaire (Block 98.2 FFQ; Block Dietary Data Systems, Berkeley, CA, USA). Patients completed the questionnaires during the baseline study period (at or within 1-week of the baseline visit). The study coordinator was available for questions regarding the FFQ, but generally patients completed the questionnaires alone. The full length (110 food items) questionnaire was designed to estimate usual and customary intake of a wide array of nutrients and food groups. This questionnaire has been validated in various studies (Block, Hartman, & Naughton, 1990; Johnson, Herring, Ibrahim, & Siega-Riz, 2007) and more recently in a sample population of Canadian women (Boucher et al., 2006). A customized version of this questionnaire, modified to reflect fortification in the Canadian diet, was used in this study (Chilibeck et al., 2013). This food-frequency has been validated for assessment of glycemic index and glycemic load (Barclay et al., 2008; Liu et al., 2001). The overall dietary glycemic load is calculated as the cumulative glycemic load of each food item multiplied by the frequency of consumption over all food items. Dietary glycemic index is calculated by dividing dietary glycemic load by the total amount of carbohydrate consumed. Each unit of dietary glycemic load represents the overall quality of carbohydrate in the diet matched to 1 gram of glucose or white bread. Completed questionnaires were scanned and analyzed to determine nutrient values (Nutrition Quest, Berkeley, CA, USA; www.nutritionquest.com). Analysis was completed in grams of macronutrient and alcohol intakes, % of energy intake, food servings (based on 2005 Food Pyramid values), and GI and GL (United States Department of Agriculture, 2005). A comparison of the 2005 USDA Food Pyramid and Eating Well with Canada's Food Guide is found in **Table 5.1**.

Table 5.1 USDA Food Pyramid and Eating Well with Canada's Food Guide

USDA Food Pyramid (2005)		
Category	**Recommendation (servings)**	
Bread, pasta, rice	6-11	
Vegetables	3-5	
Fruits, fruit juices	2-4	
Milk, cheese	2-3	
Meat, eggs, beans	2-3	
Fats, oil, sweets	Very little	
EWCFG (2011)		
Category	**Recommendation (19-50y) (servings)**	
	Female	**Male**
Vegetables and	7-8	8-10
Grain products	6-7	8
Milk/alternatives	2	2
Meat/alternatives	2	3

Comparison table of USDA Food Pyramid and Eating Well with Canada's Food guide. **y**, years. **USDA Food Pyramid,** (United States Department of Agriculture, 2005) **, EWCFG,** Eating Well with Canada's Food Guide(Health Canada, 2011).

Statistical analysis: Descriptive statistics were used to describe patient demographics and biomarker concentrations. Data are presented as mean ± standard deviation unless otherwise noted. Biomarkers are presented as median values with interquartile ranges (not normally distributed). All analysis was completed on SPSS unless otherwise noted (*IBM SPSS Statistics for Windows*, 2013)

A linear regression model was used to determine biomarkers performance at baseline using R (*R: A language and environment for statistical computing*, 2008)

Descriptive statistics were used to describe the nutritional assessment. Data are presented as mean ± standard deviation unless otherwise stated. A one-way ANOVA was used to detect significant differences between quartiles of glycemic index and glycemic load.

For repeated biomarker measures, generalized estimating equations (GEE) were used to test for an association between disease activity measures over time (i.e. The CDAI and PT index for the CD and UC, respectively) and predicting variables (i.e. therapy, gender, and age, along with hsCRP, FC, and Lf). The *identity* link function was used given that the dependent variables were continuous. An exchangeable correlation structure for the repeated measures was specified

in the GEE (Dohoo, Martin, & Stryhn, 2012). First, unconditional analyses were performed to evaluate associations between disease activity and each of the predictors (therapy, gender, visit number, age, hsCRP, FC, and Lf), obtaining unadjusted beta estimates with corresponding 95% confidence intervals (95% CI). The linearity assumption of the continuous independent variables was tested using a quadratic term. Then, a manual backward selection strategy was used to build two multivariate models, one for CD and another one for UC, removing variables with p-value ≥ 0.05. Variables removed from the models were evaluated as confounding factors, considering them as confounders if a 10% or more change in the estimates of the predictors in the model was observed. In the final model, independent, exponential, and autoregressive correlation structures were tested. The quasi-likelihood under the independence model criterion (QIC) was used to evaluate goodness of fit of the models. Estimates and corresponding 95% CI were reported for the final models. The repeated measure analyses were completed using the *xtgee* and *qic* commands in STATA (*Stata Statistical Software*, 2015).

5.4 Results

Baseline: During recruitment, 70 patients were screened for potential enrolment. Of these 42 participants who met the inclusion and exclusion criteria were subsequently enrolled. The cohort was split between 50% male and 50% female, with a mean age of 38.3 years, ranging between 19 and 70. Thirty-six percent of patients were receiving an ASA-agent, 38% receiving immunomodulators, and 19% of patients were receiving an anti-TNF agent, in addition to 7% not receiving any therapy. Only 19% of patients were smokers. The demographic characteristics of the total cohort are shown in **Table 5.2** as well as each specific cohort [CD;UC] **Appendix B**. The clinical characteristics of this cohort are shown in **Table 5.3**. Of the 26 CD patients, 46% had ileocolonic disease.

At enrolment, the median hsCRP concentration was within normal range 5.0 mg/L (normal 0 – 7.0 mg/L). However, the fecal biomarkers were elevated. The median FC concentration was 729 μg/g (normal <50 μg/g), and median fecal Lf concentration was 146.0 μg/mL (normal 0 - 7.24 μg/mL) (**Table 5.4**).

Table 5.2 Demographics and anthropometry of a newly diagnosed IBD cohort (n=42)

	Mean ± SD	**Range**	**Freq**	**(%)**
Age (y)	38.3 ± 12.1	19 - 70		
Gender				
Male			21	**50**
Female			21	**50**
Smoking Status				
Smoker			8	**19**
Non-smoker			31	**74**
Missing			3	**7**
Ethnicity				
Caucasian			39	**93**
Aboriginal			3	**7**
Therapy				
ASA Agent			15	**36**
Immunomodulators			16	**38**
Anti-TNF Agent			8	**19**
None			3	**7**
Anthropometry				
Weight (kg)	81.2 ± 20.8	47.3 – 129.1		
Height (cm)	172.4 ± 8.2	157.5 – 188.0		
BMI	26.9 ± 6.0	18.0 – 41.9		

Freq, frequency, **ASA,** amino-salicylic acid, **Anti-TNF,** anti-tumour necrosis factor**, y**, years, **BMI**, body

Table 5.3 Endoscopic assessment of disease location of the newly diagnosed cohort (n=42)

	CD, n=26		UC, n=16	
	Frequency	**(%)**	**Frequency**	**(%)**
Disease Location (CD)				
Ileal	8	**31**		
Ileocolonic	12	**46**		
Colonic	6	**23**		
Disease Location (UC)				
Pan-colonic			10	**63**
Left-sided			4	**25**
Proctosigmoditis			2	**13**

Disease location of 42 newly diagnosed IBD patients (n=42), divided into Crohn's disease (n=26) and ulcerative colitis (n=16).

Table 5.4 Biomarker characteristics in a newly diagnosed cohort (n=42)

	Median	**IQR**	**Q1 - Q3**	**Normal Range**
Biomarker				
hsCRP (mg/L)	5.0	17.0	2.0 - 19.0	0 - 7.0
FC (µg/g)	729	1126	330.3 - 1455.8	< 50
Lf (µg/mL)	146.0	290.0	6.0 - 298.0	0 - 7.24

Results of biomarker concentrations in full cohort of 42 IBD patients. Results are expressed as median values. One value was missing from hsCRP analysis. **IQR**, Interquartile range, **Q1**, quartile 1, **Q3**, quartile 3, **hsCRP,** high sensitivity C-reactive protein, **FC,** calprotectin, **Lf**, lactoferrin.

The anthropometric characteristics of the newly diagnosed cohort are found in **Table 5.2.** The mean weight of the cohort was 81.2 ± 20.8 kg and height was 172.4 ± 8.2 cm. BMI for the newly diagnosed cohort was 26.9 ± 6.0, falling into the "overweight" category (National Institute of Health, 2015).

The box and whisker plots shown in **Figures 5.1 - 5.4** display the relationship between disease activity and biomarker concentration. When stratified in relation to clinical and endoscopic disease activity of CD (CDAI, HBI and SES-CD), median values of FC, Lf, and hsCRP increase with increases in disease severity (**Figure 5.1, 5.2).** This trend is also observed between biomarkers and disease activity (pMayo, Full Mayo) in UC **(Figure 5.3, 5.4).**

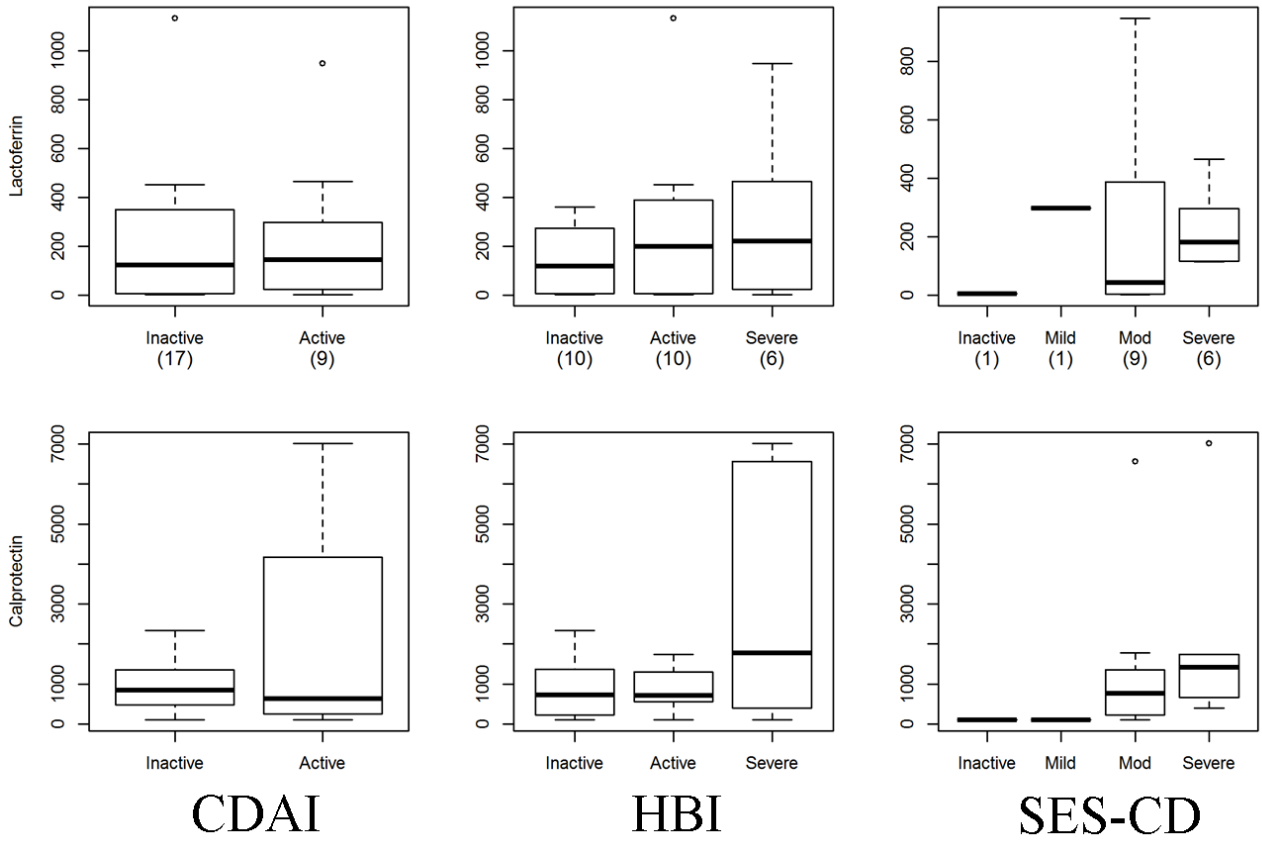

Figure 5.1 Fecal biomarkers, calprotectin and lactoferrin, stratified by disease activity in Crohn's disease. (**CDAI:** active > 150, inactive > 150, **HBI:** remission ≤ 2, active 3-7, severe ≥ 8 **SES-CD:** inactive 0-2, mild 3-6, moderate 7-15, severe ≥ 16). **CDAI,** Crohn's disease Activity Index, **HBI**. Harvey Bradshaw Index, **SES-CD,** Simple Endoscopic Score for Crohn's disease.
[o] indicates an outlier.

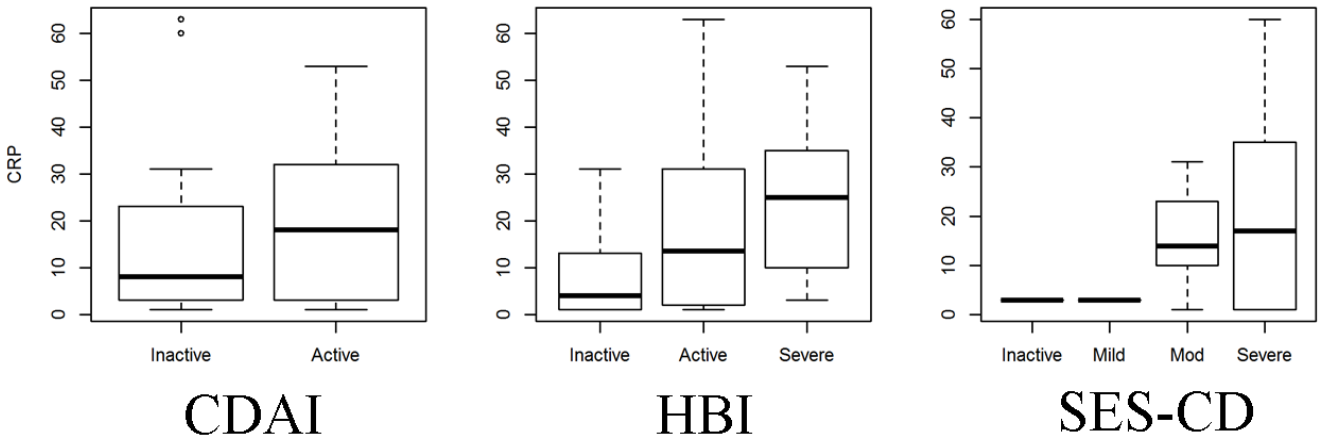

Figure 5.2 High sensitivity C-reactive protein stratified by disease activity in Crohn's disease (**CDAI:** active > 150, inactive > 150, **HBI:** remission ≤ 2, active 3-7, severe ≥ 8 **SES-CD:** inactive 0-2, mild 3-6, moderate 7-15, severe ≥ 16). **CDAI,** Crohn's disease Activity Index, **HBI**. Harvey Bradshaw Index, **SES-CD,** Simple Endoscopic Score for Crohn's disease, **CRP**, C - reactive protein. ^{o} indicates an outlier.

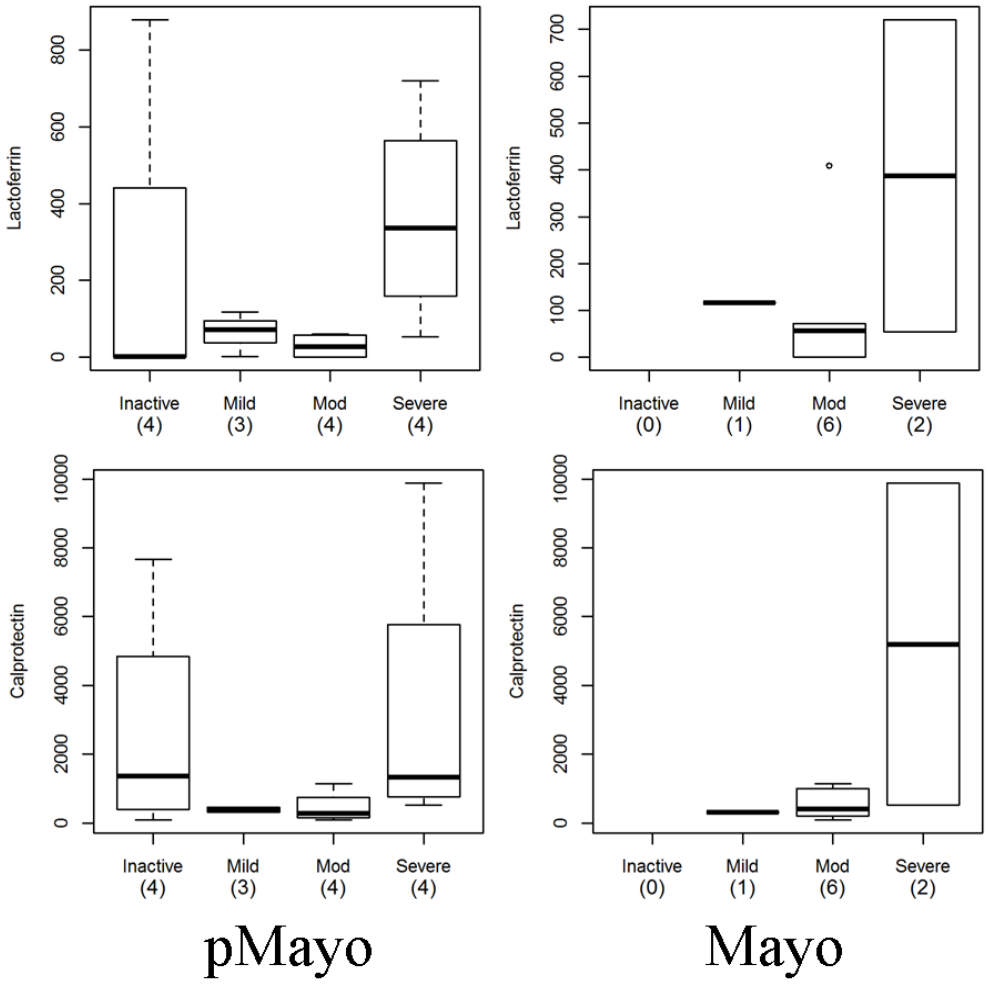

Figure 5.3 Fecal biomarkers, calprotectin and lactoferrin, stratified by disease activity in Ulcerative colitis. (**partial Mayo (pMayo)**: inactive ≤ 2, mild 3-4, moderate, 5-6, and severe 7-9. **Mayo:** inactive ≤ 2, mild 3-5, moderate 6-10, severe 11-12). **pMayo,** partial mayo score. ^{o} indicates an outlier.

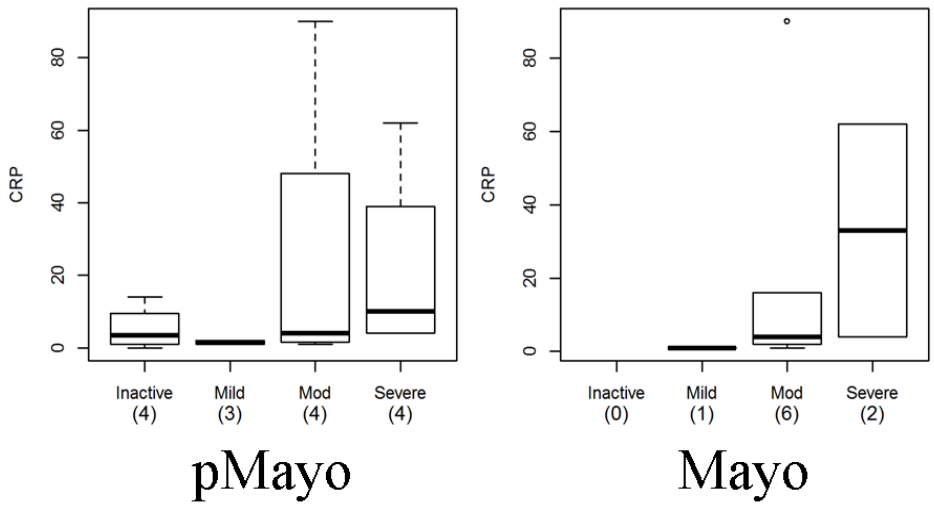

Figure 5.4 High sensitivity c-reactive protein stratified by disease activity in Ulcerative colitis (**partial Mayo (pMayo)**: inactive ≤ 2, mild 3-4, moderate, 5-6, severe 7-9 , **Mayo:** inactive ≤ 2, mild 3-5, moderate 6-10, severe 11-12). **pMayo,** partial mayo score.

The relationship between biomarkers and disease activity scores are presented by a simple linear regression found in **Table 5.5.** In CD, a modest correlation between the biomarkers and the markers of disease activity was found, with the strongest relationship between FC and SES-CD (r = 0.62). This relationship was not as strong with CRP or lactoferrin. In UC, the strongest relationship was also shown to be between FC and the Full mayo score (r = 0.61). **Figure 5.5** displays the relationship between BMI, biomarkers and glycemic index and glycemic load.

Table 5.5 Simple linear modelling scores in a newly diagnosed IBD cohort (n=42)

	FC (µg/g)		**Lf (µg/mL)**		**hsCRP (mg/L)**	
	r	p-value	r	p-value	r	p-value
CD, n=26						
HBI	**0.36**	0.0810	**0.10**	0.6407	**0.41**	<0.05
CDAI	**0.31**	0.1357	**0.13**	0.5242	**0.32**	0.1133
SES-CD, n=17	**0.62**	<0.01	**0.37**	0.1415	**0.13**	0.6283
UC, n=16						
PTI	**0.20**	0.4844	**0.30**	0.2649	**0.29**	0.3009
Partial Mayo	**0.21**	0.4744	**0.54**	<0.05	**0.43**	0.1249
Full Mayo, n=9	**0.61**	<0.01	**0.36**	0.3364	**0.48**	0.1950

Results of a linear regression model in a full cohort of 42 newly diagnosed IBD patients**.** **hsCRP**, high sensitivity C-reactive protein, **FC**, calprotectin, **Lf**, lactoferrin, **CD**, Crohn's disease, **UC**, Ulcerative colitis, **HBI**, Harvey Bradshaw Index, **CDAI**, Crohn's disease Activity Index, **SES-CD**, Simple Endoscopic Score - Crohn's disease, **PTI**, Powell Tuck Index.

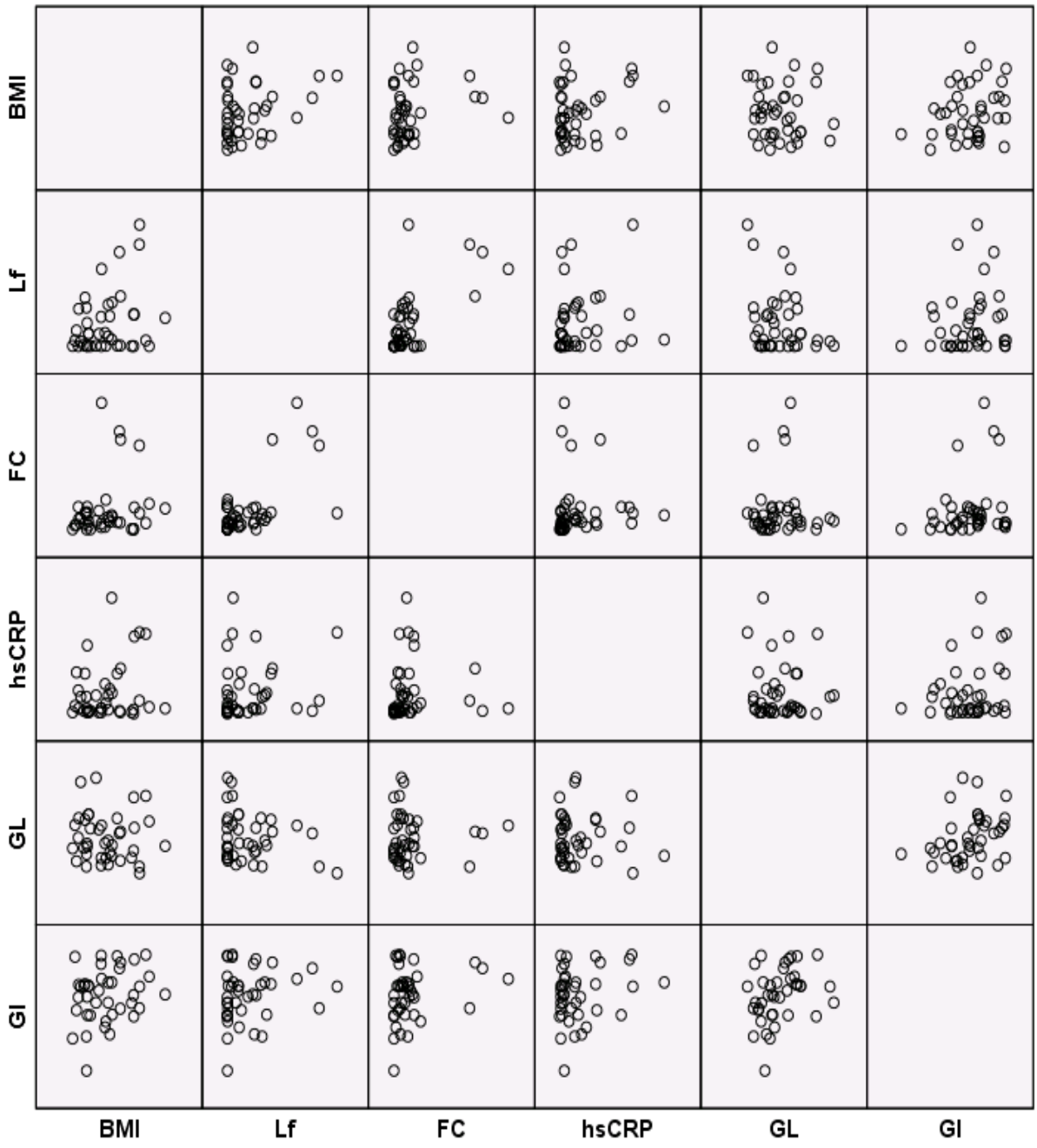

Figure 5.5 Correlation between biomarkers, body mass index (BMI) and measures of carbohydrate quality, glycemic index (GI) and glycemic load (GL) in a newly diagnosed IBD cohort (n=41). **Lf,** lactoferrin, **FC,** fecal calprotectin.

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Table 5.6 Mean daily intake of nutrients in newly diagnosed IBD (n=41)

	CD, n=25		UC, n=16		All, n=41		
	Mean (SD)	**Range**	**Mean (SD)**	**Range**	**Mean (SD)**	**Range**	**AMDR**
Energy (kcal)	1556 (545)	637 – 2750	1761 (683)	527 – 3139	1636 (603)	527 - 3139	
Protein (g)	61 (25)	20 – 118	66 (31)	6 – 144	63 (27)	6 – 144	
% of NRG	**16 (3)**	11 – 25	**15 (4)**	5 – 21	**15 (3)**	5 – 25	**10 – 35**
Fat (g)	67 (28)	21 – 124	74 (31)	11 – 140	70 (29)	11 – 140	
% of NRG	**38 (7)**	18 – 53	**37 (6)**	19 – 43	**37 (6)**	18 – 53	**20 – 35**
Carbohydrate (g)	175 (61)	76 – 356	205 (81)	104 – 387	187 (70)	76 – 387	
% of NRG	**46 (9)**	32 – 78	**48 (9)**	31 – 79	**47 (9)**	32 – 79	**45 – 65**
Alcohol (%)	4 (5)	0 – 17	4 (5)	0 - 14	4 (5)	0 – 17	
Fibre (g)	15 (8)	6 – 38	14 (9)	2 - 39	15 (8)	2 – 39	
GL	85 (33)	37 – 179	104 (39)	51 - 186	92 (36)	37 – 186	
GI	53 (4)	42 - 59	55 (3)	50 - 59	53 (4)	42 – 60	

Mean daily nutrient intake for 38 newly diagnosed (n=38) IBD patients. Data are expressed as mean (standard deviation) or as stated. **NRG,** energy, **GL**, glycemic load based on glucose standard, **GI,** glycemic index based on glucose standard, **CD,** Crohn's disease**, UC,** Ulcerative colitis, **AMDR,** acceptable macronutrient distribution range (Institute of Medicine (U.S.) & Institute of Medicine (U.S.), 2005)

Table 5.7 Mean daily food servings intake in newly diagnosed IBD (n=41)

Serving group	CD, n=25	UC, n=16	All, n=41	Food Pyramid, 2005
Vegetable	3.6 ± 2.9 (0.6 – 11.5)	2.6 ± 1.8 (0.2 – 6.6)	3.2 ± 2.5 (0.2 – 11.5)	3 – 5
Grain	3.0 ± 1.7 (0.8 – 6.3)	3.7 ± 1.7 (0.1 – 6.8)	3.3 ± 1.8 (0.1 – 6.8)	6 – 11
Meat	1.7 ± 0.9 (0.4 – 4.4)	1.8 ± 1.1 (0.2 – 4.8)	1.7 ± 1.0 (0.2 – 4.8)	2 – 3
Dairy	1.3 ± 0.9 (0.0 – 3.5)	1.5 ± 1.0 (0.1 – 3.7)	1.4 ± 0.9 (0.0 – 3.7)	2 – 3
Fruit	1.5 ± 1.1 (0.2 – 4.0)	1.4 ± 1.1 (0.1 – 4.0)	1.5 ± 1.1 (0.1 – 4.0)	2 – 4
Fat	2.8 ± 1.5 (0.5 – 5.8)	3.1 ± 1.3 (1.0 – 5.9)	2.9 ± 1.4 (0.5 – 5.9)	Very little

Mean daily food serving intake for 41 newly diagnosed IBD patients. Data are expressed as mean ± standard deviation (range) Compared with reference values from the 2005 USDA Food Pyramid (United States Department of Agriculture, 2005). CD vs. UC samples were compared using independent t-test (no significant differences were found).

An assessment of the mean daily nutrient intake for this newly diagnosed cohort is found in **Table 5.6.** The energy intake for this cohort was 1636 kilocalories (kcal) per day. The intake of macronutrients and alcohol (expressed as percentage of energy intake) was 15% dietary protein, 37% dietary fat, 47% dietary carbohydrate, and 4% from alcohol. Only dietary fat was found to be slightly outside the acceptable macronutrient distribution range (AMDR) (Institute of Medicine (U.S.) & Institute of Medicine (U.S.), 2005), although carbohydrate was close to the lower end. Total dietary fibre was calculated to be 15 grams per day for the entire cohort, which when adjusted for energy intake, was 9 g/1000 kcal/day, lower than recommended (14 g/1000 kcal/d) (Institute of Medicine (U.S.) & Institute of Medicine (U.S.), 2005). In the full cohort, GI was calculated as 53, GL as 85. In CD, intake of macronutrients and alcohol was 16% protein, 38% fat, 46% carbohydrates, and 4% alcohol. GI was calculated at 55, GL was calculated at 104. In UC, intake of macronutrients and alcohol was 16% protein, 37% fat, 48% carbohydrates, and 4% alcohol. Dietary fibre was calculated to be 14 g/1000 kcal/d, same as the recommendation. GI was calculated at 53, GL was calculated at 92.

The mean daily food serving intakes of the newly diagnosed cohort is found in **Table 5.7.** The entire cohort was found to consume less grain (3.3 ± 1.8 vs. 6-11), meat (1.7 ± 1.0 vs. 2-3), dairy (1.4 ± 0.9 vs. 2-3) and fruit (1.5 ± 1.1 vs. 2-4) compared with 2005 Food Pyramid recommendations (n=41). Vegetable consumption was within recommendations (3.2 ± 2.5 vs. 3-5). Although not statistically significant, vegetable intake was higher in CD patients compared with UC (3.6 ± 2.9 vs. 2.6 ± 1.8), with the opposite trend being seen with grain intake (3.0 ± 1.7 vs. 3.7 ± 1.7, respectively). Meat, dairy, fruit and fat servings were roughly equal (**Table 5.7)**.

Nutritional assessment and BMI values for individual CD patients, separated by male and female, are found in **Table 5.8** (n=26, 14 female, 12 male). Male BMI values were higher than females, 27.8 to 24.7, respectively. Both male and female CD patients were above the recommended dietary allowance (RDA) for protein intake (0.8 g/kg/d) at 0.9 and 0.8 g/kg/d, respectively (Institute of Medicine (U.S.) & Institute of Medicine (U.S.), 2005). However, both female and male patients fell short of fibre requirements (14 g/ 1000 kcal/d) at 9 and 12 g/1000 kcal/d, respectively (Institute of Medicine (U.S.) & Institute of Medicine (U.S.), 2005).

Nutritional assessment and BMI values for individual UC patients, separated by male and female, are found in **Table 5.9** (n=16, 6 female, 10 male). Male and female BMI values were similar, 28.5 and 28.0, respectively. Female UC patients were below the RDA for protein intake at 0.6 g/kg/day, while male patients were above at 0.9 g/kg/day. (Institute of Medicine (U.S.) & Institute of Medicine (U.S.), 2005). Similar to CD patients, both female and male patients fell short of fibre requirements at 8 and 7 g/1000 kcal, respectively (Institute of Medicine (U.S.) & Institute of Medicine (U.S.), 2005)

Table 5.8 Nutritional assessment and BMI in a newly diagnosed cohort of female CD patients (n=26)

Patient	Age	Gender	BMI	NRG (kcal)	PRO (g)	PRO (g/kg/d)	CHO (g)	FAT (g)	FIB (g)	FIB (g/1000kcal)
CD3	22	F	20.1	2750	104	2.1	356	108	28	10
CD4	19	F	19.0	1003	39	0.8	116	43	6	6
CD5	52	F	35.3	655	28	0.3	76	29	8	12
CD10	48	F	33.2	1067	35	0.4	126	50	8	7
CD11	47	F	19.5	2069	80	1.4	244	92	21	10
CD18	20	F	21.7	1575	63	1.2	170	64	13	8
CD19	25	F	25.3	637	20	0.3	92	22	7	11
CD20	22	F	21.6	1231	43	0.7	116	65	14	11
CD21	34	F	22.1	2003	68	1.0	247	84	12	6
CD22	60	F	26.6	1146	54	0.7	144	40	20	18
CD23	28	F	19.5	1861	84	1.4	187	86	12	7
CD24	32	F	21.8	1389	52	0.9	166	60	9	6
CD25	52	F	30.3	963	45	0.6	122	34	12	12
CD26	32	F	29.5	1977	68	0.9	223	93	16	8
FEMALE	**35.2**	**14**	**24.7**	**1452**	**56**	**0.9**	**170**	**62**	**13**	**9**
TOTAL	**35.9**	**26**	**26.1**	**1556**	**61**	**0.8**	**176**	**67**	**15**	**10**

Baseline demographics in 26 newly diagnosed Crohn's disease (CD) patients. Summary rows (**FEMALE** etc.) present mean data. **BMI,** body mass index, **NRG,** energy, **PRO,** dietary protein, **FAT,** dietary fat, **CHO,** dietary carbohydrate, **FIB,** dietary fibre, **n/a**, not applicable.

Table 5.8 Nutritional assessment and BMI in a newly diagnosed cohort of male CD patients (n=26) (**CONTINUED**)

Patient	Age	Gender	BMI	NRG (kcal)	PRO (g)	PRO (g/kg/d)	CHO (g)	FAT (g)	FIB (g)	FIB (g/1000kcal)
CD1	44	M	21.6	1670	73	1.0	175	59	13	8
CD2	25	M	26.3	2029	101	1.1	196	84	22	11
CD6	32	M	18.0	1027	32	0.6	201	21	38	37
CD7	33	M	24.8	1748	63	0.8	209	74	14	8
CD8	52	M	27.6	1864	118	1.2	172	70	24	13
CD9	48	M	27.2	2153	76	0.8	172	104	13	6
CD12	31	M	21.2	2143	85	1.0	246	97	27	12
CD13	36	M	33.7	1259	37	0.3	147	50	11	9
CD14	35	M	35.2	954	44	0.4	102	42	10	10
CD15	28	M	33.8	n/a	n/a	n/a	n/a	n/a	n/a	n/a
CD16	52	M	33.9	2127	61	0.6	197	124	14	7
CD17	24	M	30.4	1599	55	0.6	186	74	12	7
MALE	**36.7**	**12**	**27.8**	**1688**	**68**	**0.8**	**182**	**73**	**18**	**12**
TOTAL	**35.9**	**26**	**26.1**	**1556**	**61**	**0.8**	**176**	**67**	**15**	**10**

Baseline demographics in 26 newly diagnosed Crohn's disease (CD) patients. Summary rows (**MALE** etc.) present mean data. **BMI,** body mass index, **NRG,** energy, **PRO,** dietary protein, **FAT,** dietary fat, **CHO,** dietary carbohydrate, **FIB,** dietary fibre, **n/a**, not applicable.

Table 5.9 Nutritional assessment and BMI in a newly diagnosed cohort of UC patients (n=16)

Patient	Age	Gender	BMI	NRG (kcal)	PRO (g)	PRO (g/kg/d)	CHO (g)	FAT (g)	FIB (g)	FIB (g/1000kcal)
UC1	41	F	28.2	1291	56	0.7	172	41	11	8
UC5	38	F	22.1	2003	68	1.0	247	84	12	6
UC7	43	F	41.9	1422	47	0.4	166	69	17	12
UC10	50	F	25.4	527	6	0.1	104	11	2	3
UC14	35	F	21.0	1431	69	1.1	155	64	15	10
UC15	55	F	29.6	1085	40	0.5	145	41	12	11
FEMALE	**43.7**	**6**	**28.0**	**1293**	**48**	**0.6**	**165**	**52**	**12**	**8**
UC2	35	M	36.9	2304	87	0.7	276	95	11	5
UC3	49	M	22.6	1870	54	0.9	226	79	13	7
UC4	70	M	28.1	1146	43	0.5	122	48	6	5
UC6	36	M	30.1	1776	95	1.0	189	73	17	9
UC8	45	M	33.8	3139	144	1.5	350	140	39	12
UC9	54	M	27.2	1315	51	0.7	105	63	7	5
UC11	35	M	37.7	2069	97	0.8	220	81	10	5
UC12	20	M	25.5	2035	77	0.9	212	95	12	6
UC13	34	M	18.6	1757	60	1.0	202	82	12	7
UC16	37	M	24.0	3009	64	0.9	387	117	31	10
MALE	**41.5**	**10**	**28.5**	**2042**	**77**	**0.9**	**229**	**87**	**16**	**7**
TOTAL	**42.3**	**16**	**28.3**	**1761**	**66**	**0.8**	**205**	**74**	**14**	**8**

Baseline demographics in 16 newly diagnosed Ulcerative colitis (UC) patients. Summary rows (**FEMALE** etc.) present mean data. **BMI,** body mass index, **NRG,** energy, **PRO,** dietary protein, **FAT,** dietary fat, **CHO,** dietary carbohydrate, **FIB,** dietary fibre

A one-way ANOVA was used to determine the relationship between quartiles of GI and GL (based on glucose standard) and fecal biomarkers, FC and Lf (presented as median values). The concentration of Lf and FC increased across quartiles of glycemic index (µg/mL and µg/g), respectively, from the first quartile (n=10, 3.5 and 420.0), to the second (n=11, 695.0 and 91.0), third (n=10, 868.5 and 117.0) and fourth (n=10, 1172.0 and 163.5) (**Figure 5.6**). The concentration of Lf and FC did not follow the same trend for quartiles of glycemic load (µg/ml and µg/g), respectively, from the 1st quartile (n=10, 118.5 and 776.5), to the second (n=11, 61.0 and 612.0), third (n=10, 210.0 and 1665.5) and fourth (n=10, 49.0 and 676.0) (**Figure 5.7**). Although a clear trend is observed between quartiles of GI, no statistically significant differences in biomarkers between these quartiles

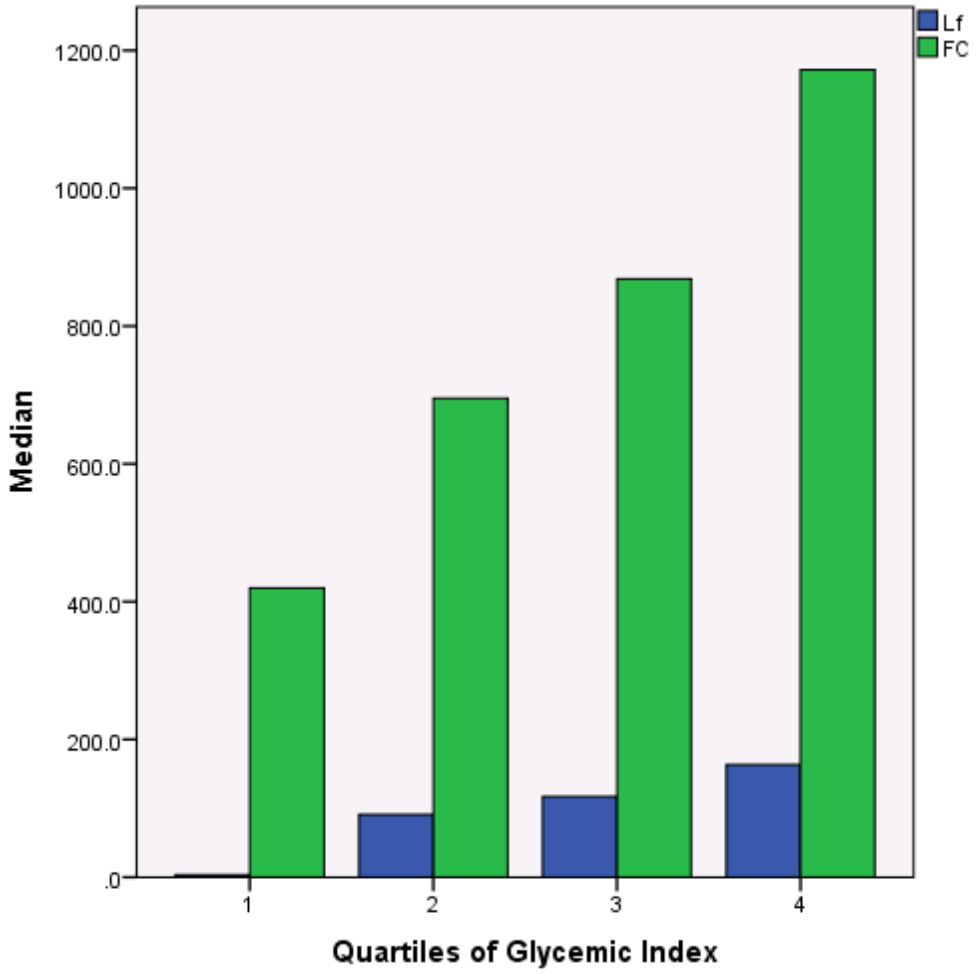

Figure 5.6 Relationship between glycemic index (GI) and fecal biomarkers, lactoferrin (blue, µg/mL) and calprotectin (green, µg/g) across quartiles in newly diagnosed IBD cohort (n=41). Biomarkers are presented as median values. **Lf,** lactoferrin, **FC,** fecal calprotectin.

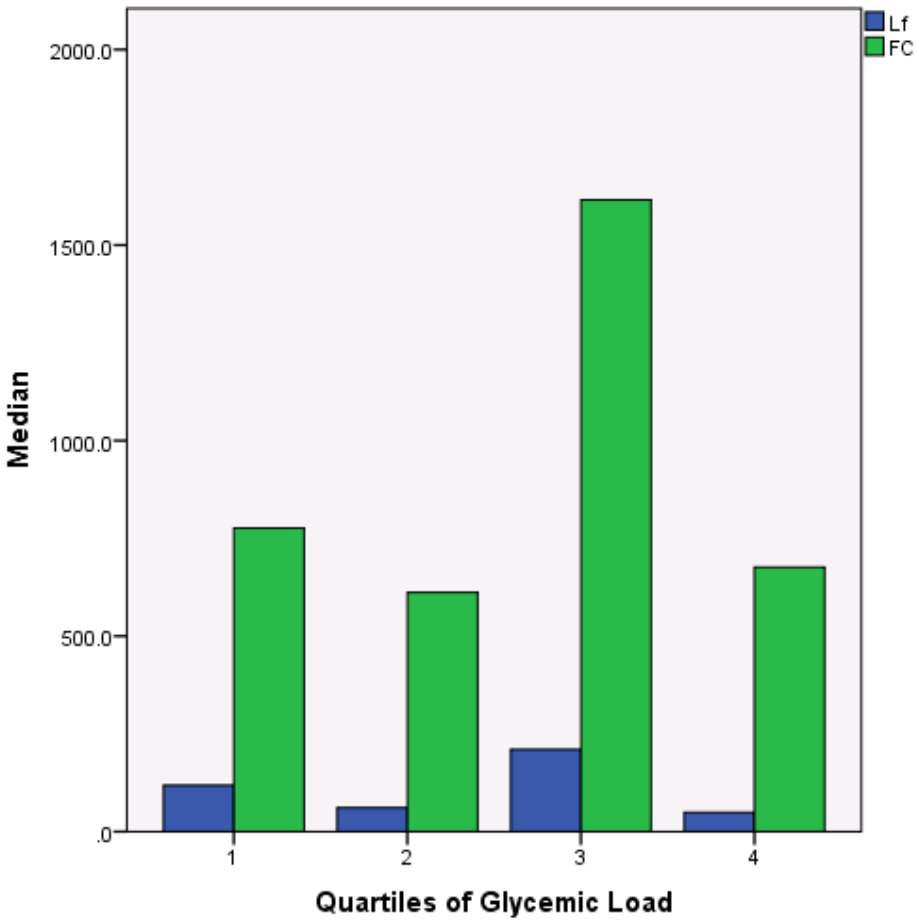

Figure 5.7 Relationship between glycemic load (GL) and fecal biomarkers, lactoferrin (blue, µg/ml) and calprotectin (green, µg/g) across quartiles in newly diagnosed IBD cohort (n=41). Biomarkers are presented as median values. **Lf,** lactoferrin, **FC,** fecal calprotectin.

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Repeated Measures: For prediction of CDAI among patients with CD, our analyses identified therapy (p-value = 0.006), hsCRP (p-value = <0.001), and FC (p-value = <0.001) as significant predictors of disease activity over time (see **Table 5.10**). Age, hsCRP, and FC met the assumption of linearity (p-values≥0.05). Lf was excluded during unconditional analysis because it was not a significant predictor (p-value = 0.157). In the model building process, therapy, age, gender, and hsCRP were removed during the backward selection process, and only FC was retained. However, hsCRP was identified as a cofounding variable (using a 10% cut-off for precision) and included in the final model. Therefore, the final model including FC and hsCRP (**Table 5.11**) fitted best (QIC=491,637.832) than the null model (QIC=1,093,062.781).

Table 5.10 Unconditional analysis for repeated measures using Crohn's disease Activity Index (CDAI) (100 observations, n=25)

	β estimates	**95% CI**		**p-value**
Therapy				
5-ASA	64.8598	1.7829	127.9367	0.044
Immuno	77.5797	28.6622	126.4972	0.002
Anti-TNF	82.4162	18.1051	146.7274	0.012
None	ref			
Gender				
Male	-10.1875	-69.9515	49.5766	0.738
Female	ref			
Age	0.4512	-2.6890	3.5913	0.778
Lf (80 obs.)	0.0965	-0.0372	0.2303	0.157
hsCRP (94 obs.)	1.8078	1.0896	2.5261	<0.001
FC (84 obs.)	0.0351	0.0224	0.0478	<0.001

Unconditional analysis of repeated measures in predicting CDAI score. 100 observations for each variable unless state otherwise. **CDAI,** Crohn's disease Activity Index, **5-ASA,** amino-salicylic acid, **Anti-TNF,** anti-tumour necrosis factor agent**, immuno,** immunosuppressant, **ref**, reference variable, **Lf,** lactoferrin, **FC**, fecal calprotectin, **hsCRP**, high sensitivity C-reactive protein, **obs.,** number of observations.

Table 5.11 A multivariate general estimating equation for all repeated measures using Crohn's disease Activity Index (CDAI) (79 observations, n=25)

	β estimates	95% CI		p-value
hsCRP	0.9576	-0.3968	2.3119	0.116
FC	0.0290	0.0144	0.0433	<0.001

General estimating equation modelling results of the predictive ability of repeated measures in predicting disease activity scores. **CDAI,** Crohn's disease Activity Index, **FC**, fecal calprotectin, **hsCRP**, high sensitivity C-reactive protein.

For prediction of PT among patients with UC, unconditional analyses identified therapy (p-value <0.001), hsCRP (p-value = 0.007), and FC (p-value = 0.002) as significant predictors. Lf was excluded during unconditional analysis, considering that it was not a significant predictor of disease activity (p-value = 0.106) (**Table 5.12**). There was no evidence that age, hsCRP, and FC violated the assumption of linearity (p-values ≥ 0.05). In the multivariable model building, therapy and age were removed during the backward selection process, and gender, FC, and hsCRP were retained. Therapy was identified as a cofounding variable and included in the final model (using a 10% cut-off for precision).

Table 5.12 Unconditional analysis for repeated measures using Powell Tuck (PT) Index (n=16, 65 observations)

	β estimates	95% CI		p-value
Therapy				
5-ASA	-0.9046	-5.7378	3.9286	0.714
Immuno	3.9944	-0.3651	8.3539	0.073
Anti-TNF	-0.8826	-5.4437	3.6784	0.704
None	ref	ref	ref	ref
Gender				
Male	2.0856	-0.1495	4.3207	0.067
Female	ref			
Age	-0.0106	-0.0647	0.0440	0.705
Lf (38 obs.)	0.0052	-0.0011	0.0115	0.106
hsCRP (56 obs.)	0.0760	0.0206	0.1314	0.007
FC (45 obs.)	0.0006	2.5067	4.7097	0.002

Unconditional analysis of repeated measures in predicting disease activity scores. 65 observations for each variable unless state otherwise. **PT,** Powell Tuck Index, **5-ASA,** amino-salicylic acid, **Anti-TNF,** anti-tumour necrosis factor agent**, immuno,** immunosuppressant, **ref**, reference variable, **Lf,** lactoferrin, **FC**, fecal calprotectin, **hsCRP**, high sensitivity C-reactive protein, **obs.,** number of observations.

The final model predicting PT among patients with UC included FC, hsCRP, gender and therapy (QIC=292.564) had a better model fit over the null model (QIC=675.992) (**Table 5.13**). The exchangeable correlation structure provided better QIC than the independent, exponential, and autoregressive structures.

Table 5.13 A multivariate general estimating equation for all repeated measures using Powell Tuck (PT) (40 observations, n=16)

	β estimates	**95% CI**		**p-value**
Therapy				
5-asa	-0.8466	-3.8463	2.1530	0.580
Immuno	1.5018	-1.3649	4.3685	0.305
Anti-TNF	-1.0300	-5.1138	3.0539	0.621
None	ref			
Gender				
Male	1.9667	0.0315	3.9019	0.046
Female	ref			
hsCRP	0.0770	0.0365	0.1176	<0.001
FC	0.0006	0.0003	0.0009	<0.001

General estimating equation modelling results of the predictive ability of repeated measures in predicting disease activity scores. **PT,** Powell Tuck index, **FC**, fecal calprotectin, **hsCRP**, high sensitivity C-reactive protein, **ref**, reference variable.

5.5 Discussion

The primary objective of this research was to develop a single-center, prospective cohort of newly-diagnosed adults with IBD, in an effort to evaluate the predictive performance of select endoscopic, serologic, and fecal biomarkers in long-term disease course. Over 5 years, 42 newly diagnosed patients were enrolled in this single-center study. The original goal for recruitment was set at 70 patients over 2-years. Poor recruitment limited the statistical power of this study. Adequate numbers of newly diagnosed patients in single research center (population ~200,000 residents) may not be feasible.

The fecal biomarkers in this study, FC and Lf, acting as surrogate markers of inflammation, fell outside the ranges of healthy levels (**Table 5.3**). The serum marker, hsCRP, was within the normal range for healthy individuals. Biomarker concentrations outside of the normal ranges are expected in an inflammatory condition like IBD (Vermeire, 2006).

Clinical measures of disease activity have been criticized for their inclusion of subjective variables. However, endoscopic measures of disease activity are invasive and expensive. Ideally, fecal biomarkers would be suitable surrogate markers of inflammation for these tests. In CD, a modest correlation between the biomarkers and the traditional endoscopic markers of disease activity was found, with the strongest relationship between FC and SES-CD ($r = 0.62$). This relationship was not as strong with CRP or lactoferrin. In UC, the strongest relationship was also shown to be between FC and the Full mayo score ($r = 0.61$). A comprehensive overview of the relationship between FC and endoscopic activity can be found in **Table 2.2** of this document. Our results are in agreement with the correlation values presented in the 19 studies reviewed in that table ($r = 0.48$ to 0.87). The results from this study are supported by the observations of others (Cellier et al., 1994; Jones et al., 2008; Kane et al., 2003). One major difference between those studies and the one presented in this paper is the study population. An advantage of this study, is that a newly diagnosed cohort presents a clearer look at the relationship between fecal biomarkers and endoscopic disease activity.

Although the sample size was small, our data suggests that the use of clinical disease activity measures in newly diagnosed IBD may be adequate for determining inflammatory

burden and for risk stratification. However, this work also confirms the lack of confidence in clinical measures of disease activity assessment.

A secondary aim of this study was to provide a nutritional and anthropometric baseline assessment in newly diagnosed patients. Our cohort had an overall BMI of 27.1, that is categorized as "overweight" (National Institute of Health, 2015). The energy intake for this cohort was calculated at 1636 kcal per day. There is a discrepancy between the reported energy intake and BMI in this patient population. Macronutrients and alcohol as expressed as percentage of energy intake was 15% dietary protein, 37% dietary fat, 47% dietary carbohydrate, and 4% from alcohol. Only dietary fat fell outside of the AMDR (Institute of Medicine (U.S.) & Institute of Medicine (U.S.), 2005). Dietary fibre was inadequate at 9 g/1000 kcal per day of intake. In both male and female IBD patients, protein intake was found to be adequate in reference to the RDA, however fibre as under the recommended amount (**Table 5.8, 5.9**).

Geerling et al. (2000) was the first study to perform a nutritional assessment at IBD diagnosis (Geerling et al., 2000). Patients that had been diagnosed with IBD in the previous 6-months were enrolled in this study. This well designed study assessed disease activity, CRP and body composition. Dietary intake was assessed by cross-check dietary history (in-home dietitian assessment cross-checked with FFQ). Of note, this was a multi-center study (3 centers) based in The Netherlands, where the sixty-nine patients came from a prospective 71 that been diagnosed in the study period (91% enrolment). Sixty-nine age- and sex-matched controls volunteered for this study. BMI was found to be 22.2 ± 2.7 in CD (n=23) and 23.1 ± 3.0 in UC (n=46) (mean ± SD), far lower than the BMI values found in our study, 26.1 and 28.3, respectively. Energy intake was calculated at 11.0 ± 3.4 MJ (2629 ± 813 kcal) for CD patients and 10.3 ± 2.6 MJ (2462 ± 621 kcal). However, the authors noted this may because the patients did not have strongly elevated disease activity, thus a higher energy intake (Geerling et al., 2000). These are significantly higher energy intakes than found in our study (**Table 5.6**). Macronutrients and alcohol were expressed as a percent of energy intake. In CD, 14.7 ± 2.2% calories came from protein, 34.0 ± 7.6% from fat, 51.0 ± 7.4% from carbohydrates and 0.9 ± 1.3% from alcohol. From the macronutrients, only carbohydrates were statistically higher in CD than controls. Compared with our findings, fat and alcohol intake was significantly lower (p-value < 0.01). In UC, 14.8 ± 2.5% calories came from protein, 35.2 ± 6.3% from fat, 49.3 ± 7.7% from

carbohydrates and 1.8 ± 3.0% from alcohol. From the macronutrients, only protein was statistically lower than controls. There were no significant differences compared with our findings. In Geerling et al. (2000), both UC and CD, alcohol consumption was statistically lower than in healthy controls. Fibre intake was calculated at 1.6 ± 0.5 g/MJ (7 g/1000 kcal/d) for CD patients and 1.9 ± 0.5 (8 g/1000 kcal) from UC patients, compared with the 10 and 8 g/1000 kcal/d for our CD and UC patients, respectively. Although they did not measure glycemic index or load, the intake of mono- and disaccharides, or simple carbohydrates, as a percent of the diet was calculated. In CD, 26.7 ± 6.6 % of energy was from simple carbohydrates, significantly more than healthy controls.

Discrepancies between these two studies are most likely due to the differences in dietary intake assessment. In our study, we used a FFQ, assessing the usual consumption of a list of foods, compared with the cross-check dietary history used by Geerling et al. (2000). FFQ's capture a variety of information, although at differing levels of accuracy. They are inexpensive and feasible to administer in studies where multiple food records or 24-hour food recalls would not be possible (Liu, 1994). Additionally, they are appropriate to capture the usual intake of the individual, usually retrospectively before a disease develops (Nutrition Quest, 2015). One limitation is the recall bias introduced into our study by using a FFQ to retrospectively capture dietary information (Molodecky, Panaccione, Ghosh, Barkema, & Kaplan, 2011)

In this study, our IBD cohort was found to consume less grain, meat, dairy and fruit compared with 2005 Food Pyramid recommendations (**Table 5.7)**. Also, as previously noted, comparatively our cohort BMI was high and energy intake was low, that may highlight an underestimation of food intake by the FFQ.

In our study, when energy intake was separated into quartiles (not shown in results), the most striking observation was that the lowest quartile of energy had the highest intake of carbohydrates at 55 ± 13.7%. As energy increased across quartiles, this intake dropped to 44.5 ± 5.9%, 44.7 ± 4.3%, and 43.7 ± 6.1%. At lower intakes, patients have higher carbohydrates, presumably because they are sicker. However, it is unknown if this is the result of the disease instead of the cause (Riordan et al. 1998).

Another component of our secondary aim was evaluating GI and GL, or "carbohydrate quality", in this newly diagnosed cohort, along with assessing the relationship between these measures and fecal biomarkers. Using the glucose standard, GI and GL was measured in our full cohort at 53 and 92, respectively. Healthy control GI and GL values of 51 and 120, respectively, has been reported elsewhere (n=1503) (Lahmann et al., 2014). The difference in these GI values are statistically significant (p-value<0.01). Although not statistically significant, a clear trend was observed between GI (measured by glucose standard) and the median concentration of Lf and FC (**Figure 5.6).**

Furthermore, this study aimed to observe the predictive nature of biomarkers through disease course in newly diagnosed IBD. The biomarkers, hsCRP, FC and Lf, along with gender, therapy and age were studied as predictors of disease activity. In CD, a final model for predicting CDAI included hsCRP and FC (**Table 5.11**). The beta (β) estimates for hsCRP and FC were calculated at 0.9576 and 0.0290, respectively. Meaning for every 1 unit increase in the hsCRP and FC, this model predicts CDAI to increase by 0.9576 and 0.0290 points, respectively. In UC, a final model for predicting PT among patients included FC, hsCRP, gender and therapy (**Table 5.13**). The β estimates for therapy were calculated at -0.8466 (5-ASA), 1.5018 (immunomodulators), -1.0300 (anti-TNF agents) with no therapy acting as the reference category. Thus, PT scores decreased when 5-asa and anti-TNF therapy were used as therapy. Also, the β estimates for hsCRP and FC were calculated at 0.0770 and 0.0006, respectively, showing that biomarkers have a smaller impact on PT score estimation, although PT scores are smaller values. The clinical scores, CDAI and PT, were used as outcomes due to low number of endoscopic measures. These results agree with the significance of FC and hsCRP in the prediction of CDAI found in the previous chapter (**Chapter 4**) of this thesis. The results from the building of these models are supported by others studying the predictive nature of biomarkers on disease activity (Björkesten et al., 2012; Langhorst et al., 2008).

5.6 Conclusion

This study observed a strong relationship between biomarkers and disease activity in newly diagnosed disease activity. The biomarker, FC, was found to be consistently related to measures of disease activity at baseline, particularly endoscopic measures. Both hsCRP and FC were found to predict disease activity through newly diagnosed disease course. The major food groups and dietary fibre intake were shown to be lower in this patient population compared with reference intakes. However, protein intake was shown to be adequate. Lastly, GI has a strong, positive correlation with fecal biomarkers. Fecal biomarkers have been observed to be predictive of disease activity in newly diagnosed IBD and should be included in future studies as surrogate markers of inflammation whenever possible.

CHAPTER 6

CALCULATION AND FEASIBILITY OF FECAL CALPROTECTIN IN HEALTHY AND IBD PREGNANCY

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Not yet published

IBD has a peak incidence between 18 – 35 years of age, making management during pregnancy very common. The gold standard for assessing disease activity is endoscopic procedures, which can be unpleasant and painful for patients, and time-consuming and expensive to perform. Even though endoscopy can be safely performed in pregnancy, patients and clinicians prefer to avoid invasive procedures during this time, making assessment through non-invasive surrogate markers desirable. FC, a neutrophil derived protein, is a promising biomarker in the study of intestinal inflammation. Studies have shown FC to be successful in distinguishing between IBD and irritable bowel syndrome, in addition to having a strong relationship with endoscopic disease activity. However, many studies have excluded the recruitment of pregnant patients. The objective of this study was to establish FC reference values for healthy pregnant patients. Also, FC was analyzed in pregnant patients with IBD, to assess the utility of measuring this biomarker in this important clinical subpopulation. This work adds to the evidence suggesting biomarkers demonstrate utility and predictive ability in IBD.

Author role: I completed the lab analysis, most of the statistical analysis and manuscript preparation and writing for this chapter.

Calculation and feasibility in fecal calprotectin in healthy and IBD pregnancy

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Keywords: Biomarkers, Pregnancy, Inflammatory Bowel Disease, Calprotectin

6.1 Introduction

FC is a promising fecal biomarker in the study of intestinal inflammation. FC is a neutrophil-derived protein released locally in the gut in response to inflammation (Røseth, Schmidt, & Fagerhol, 1999). FC resists metabolic degradation by intestinal bacteria and is stable in stool samples for up to one week at room temperature (Røseth et al., 1992). Numerous studies have shown FC to be effective in distinguishing inflammatory vs. non-inflammatory conditions (IBD vs. IBS) with high sensitivity (93%, 95% CI 85-97%) and specificity (96%, 95% CI 79-99%) (van Rheenen et al., 2010). Additionally in patients with IBD, the concentration of FC has been shown to correlate significantly with endoscopic disease activity in both UC and CD (Schoepfer et al., 2009; Sipponen et al., 2008). Previous studies studying FC have excluded pregnant patients. The normal physiologic changes of pregnancy are known to affect biomarkers, such as hemoglobin, white blood cell and platelet counts, CRP and ESR, demonstrating the need for FC reference values in pregnancy (Belo et al., 2005; Larsson, Palm, Hansson, & Axelsson, 2008; Milman, Bergholt, Byg, Eriksen, & Hvas, 2007; van den Broe & Letsky, 2001).

6.2 Objectives

This work aims to establish reference values for FC throughout healthy pregnancy (HP). A secondary objective is to assess the utility of FC for measuring intestinal inflammation in pregnant patients with pre-existing IBD (IBDP).

6.3 Materials and methods

Patient recruitment: HP patients were recruited as part of a prospective, observational cohort study. Patients were recruited from obstetric clinics in the Saskatoon Health Region, Saskatchewan. Pregnant women greater or equal to 18 years of age with no history of gastrointestinal disorders were eligible for the study. Patients with a history of IBD, gastrointestinal bleeding, gastrointestinal neoplasia, familial adenomatous polyposis and hereditary nonpolyposis colorectal cancer syndromes, history of colonic surgery, and intake of nonsteroidal anti-inflammatory drugs, aspirin and anticoagulants within the previous month were excluded. IBDP were recruited as part of a prospective, observational cohort by gastroenterologists from the Multidisciplinary Inflammatory Bowel Disease Clinic (MDIBDC) at

the University of Saskatchewan. Pregnant women greater or equal to 18 years of age, with IBD (based on standard clinical, radiologic, endoscopic, and histologic criteria) were eligible for the study.

Data collection: Baseline demographic (age, smoking status) and medical information (comorbid medical conditions, medications) was recorded. Gastrointestinal symptoms (abdominal pain, stool frequency, stool consistency using the Bristol Stool Scale, presence of rectal bleeding) (Lewis & Heaton, 1997) in the 3 days prior to stool sample collection was recorded. Disease activity was assessed at baseline and at each clinic visit using the Simple Clinical Colitis Activity Index for UC and the Harvey Bradshaw Index for CD (Harvey & Bradshaw, 1980; Walmsley, Ayres, Pounder, & Allan, 1998). Blood work was completed as per routine care, and if clinically indicated, endoscopic and radiologic tests were performed. Fecal samples were obtained during each trimester and within 1 - 6 months post-partum. Longitudinal assessment of FC was performed throughout pregnancy during each trimester (Trimester 1 [T1]: 1-13 weeks, Trimester 2 [T2]: 14-26 weeks, Trimester 3 [T3]:27-40 weeks) and within 6 months post-partum. This study was approved by the University of Saskatchewan's Biomedical Research Ethics Board (Bio-REB) (Bio# 13-194, 13-238).

Calprotectin analysis: FC was determined using a Quantum Blue® FC High Range Rapid Test using the Quantum Blue Reader® point-of-care (POC) desk-top reader (ALPCO Immunoassays, Salem, NH). Approximately, 30 mg of fecal sample was pressed into a base cap and fitted on the extraction tube. This device was filled with 4 mL of extraction buffer and homogenized by vortex for 1 minute. A 1:16 dilution was performed with chase buffer and 80 µL was positioned into the test cartridge for analysis. Each run took 15 minutes to complete and results were displayed in micrograms per gram (µg/g) on the POC display. Samples from HP patients were run with Rapid Test kits (30 - 300 µg/g) with a quantitation limit (QL) of 30 µg/g. Samples from IBDP patients were analyzed with High Range test kids (100 - 1800 µg/g) with a QL of 100 µg/g. Parameters that were detectable but below the QL were assigned a value of half the QL (15 µg/g for HP, 50 µg/g for IBDP).

Statistics: Descriptive statistics were used to describe patient demographics and FC concentrations. Data is presented as mean ± standard deviation (SD), unless otherwise noted**.**

Since biomarkers are not normally distributed, some results are presented as medians with interquartile ranges (IQR). The Mann-Whitney U test was used to identify significant median differences in the FC values between the healthy and IBD pregnant patients. The Friedman's ANOVA test was used to determine rank differences across the four FC measures (T1-T3, as well as post-partum). Wilcoxon Matched-Pairs Signed Rank tests were also used to identify median differences between the four measurements of FC. Statistical analyses were performed using the IBM SPSS Statistics software, version 23 (SPSS Inc. Chicago, IL).

6.4 Results

Between February 2014 and June 2015, over 80 pregnant patients were screened for potential enrolment and 46 healthy pregnant participants who met the inclusion and exclusion criteria were enrolled. Seventeen patients withdrew from the study. Similarly, 15 patients with established IBD were enrolled. Three IBD patients withdrew from the study. The average age in the healthy pregnant cohort was 33.4 ± 4.0 years. In the HP group, 121 FC samples were collected. The average age of the IBD pregnancy was 30.5 ± 3.9 years. In the IBD group, 35 FC samples were collected. Seventy-eight percent of healthy pregnant patients reported never smoking, with 15% being former and 4% current smokers (**Table 6.1**). Seventy-three percent of IBD pregnancy patients reported never smoking, while the remaining 27% reported as former smokers. In the IBD group, 60% had established CD, with 40% having UC (**Table 6.1**). Baseline bowel habits were assessed. The majority of HP patients reported no issues with bowel habits at baseline (98% had no abdominal pain or rectal bleeding). More than two thirds of IBD pregnancy patients reported no abdominal pain or rectal bleeding.

Table 6.1 Healthy and IBD pregnant patient demographics (n=46, n=15, respectively)

	Healthy Pregnancy (n=46)			**IBD pregnancy (n=15)**		
	Mean ± SD (range)	**Frequency**	**(%)**	**Mean ± SD (range)**	**Frequency**	**(%)**
Age	33.4 ± 4.0 (24 - 44)			30.5 ± 3.9 (25 - 38)		
FC samples		121			35	
Smoking Status						
Never		36	**78**		11	**73**
Former		7	**15**		4	**27**
Current		2	**4**		0	**0**
Missing		1	**2**		0	**0**
Disease Type						
CD					9	**60**
UC					6	**40**

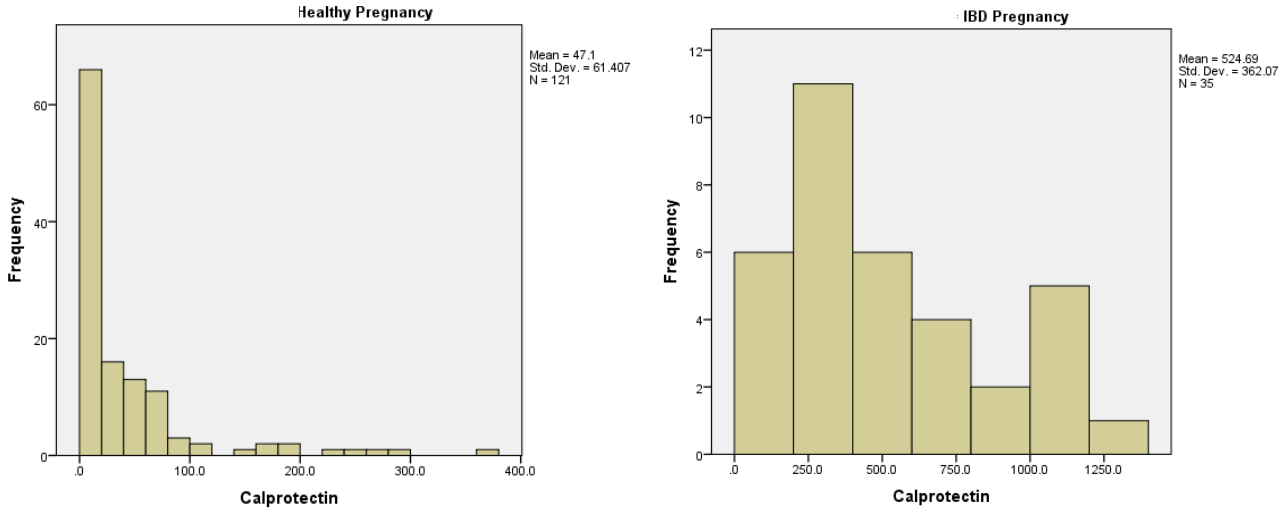
Baseline demographics in 46 healthy and 15 IBD pregnant patients. **FC**, fecal calprotectin, **wk**, weeks, **CD**, Crohn's disease, **UC**, ulcerative colitis.

In healthy pregnancy, FC values below the limit of detection were found across each assessment period, with an IQR of 37, 30, 44, and 115 for T1-T3 and post-partum, respectively (**Table 6.2)**. The overall median for HP was 15 µg/g with an IQR of 38.

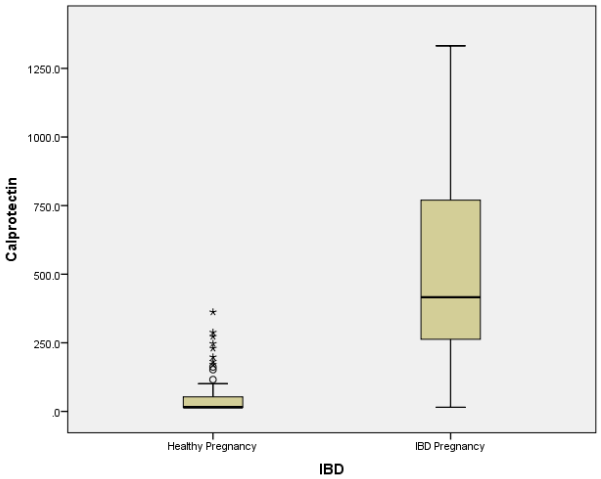
In IBD pregnancy, median FC values of 477, 337, 469, and 439 µg/g were found in T1-T3 and post-partum, respectively. The IQR for T1-T3 were 571, 857, and 281, with no IQR for the postpartum measurement in this group (**Table 6.3**). The overall median for IBDP was 416 µg/g with an IQR of 516. Results of the non-parametric analysis in IBD patients are not shown since comparisons cannot be made.

Results of the non-parametric analyses for HP patients throughout pregnancy and post-partum are shown in **Table 6.4 (**n=12**).** The Friedman's ANOVA test did not identify significant differences across the four measurements, $\chi^2(3)=1.657$, $p=0.647$. The pair-wise comparisons, Wilcoxon Matched-Pairs Signed Rank tests, showed no significant differences among the FC measures (T1-T3, and post-partum medians) (**Table 6.5)**. **Figure 6.2** presents box-plots of the four measures.

The Mann-Whitney U test identified significant median differences in the calprotectin values between the healthy and IBD pregnancy patients ($U=203.5$, $z=-8.474$, $p<0.001$), see **Figure 6.1(b)**.



(a)



(b)

Figure 6.1 (a) Histograms of fecal calprotectin for the healthy and IBD group. (b) Box plots depicting different distributions of calprotectin levels of the healthy pregnancy and IBD groups. The circles and asterisks represent outliers and extreme outliers, respectively.

Table 6.2 Fecal calprotectin in healthy pregnant patients (n=46)

	Trimester 1 (n=46)	Trimester 2 (n=37)	Trimester 3 (n=24)	Postpartum (n=14)	Total (n=121)
Median	15	15	15	15	15
Mean ± SD	44 ± 59	44 ± 57	38 ± 36	81 ± 99	47 ± 61
Range	15 - 362	15 - 273	15 - 152	15 - 288	15 - 288
IQR	37	30	44	115	38

Fecal calprotectin measurements in healthy pregnant patients. Analysis limit of detection was <30 µg/g. Readings of <30 µg/g (below detection limit), <50 µg/g (no inflammation), 50 - 200 µg/g (mild inflammation), >200 µg/g (active organic disease). **SD**, standard deviation, **IQR**, interquartile range

Table 6.3 Fecal calprotectin in IBD pregnant patients (n=12)

	Trimester 1 (n=12)	Trimester 2 (n=13)	Trimester 3 (n=9)	Postpartum (n=1)	Total (n=35)
Median	477	337	469	439	416
Mean ± SD	537 ± 383	535 ± 436	504 ± 262	439	525 ± 362
Range	15 - 477	50 - 1180	183 - 1082		15 – 1180
IQR	571	857	281		516

Fecal calprotectin measurements in IBD pregnant patients. Analysis limit of detection was <100 µg/g. Readings of <30 µg/g (below detection limit), <50 µg/g (no inflammation), 50 - 200 µg/g (mild inflammation), >200 µg/g (active organic disease). **SD**, standard deviation, **IQR**, interquartile range.

Table 6.4 Non-parametric fecal calprotectin statistics during health pregnancy (n=12)

	Mean (µg/g)	SD	Range	Median	Mean Rank[a]
1st Trimester	24	19	15 – 78	15	2.21
2nd Trimester	30	18	15 – 64	23.5	2.5
3rd Trimester	38	42	15 – 152	15	2.63
Post-partum	51	69	15 – 247	15	2.67

Fecal calprotectin measurements in HP pregnanct patients. Analysis limit of detection was <100 µg/g. Readings of <30 µg/g (below detection limit), <50 µg/g (no inflammation), 50 - 200 µg/g (mild inflammation), >200 µg/g (active organic disease). **SD**, standard deviation. a. Friedman Test Ranks.

Table 6.5 Wilcoxon Signed Ranks Test among healthy pregnancy trimesters (n=12)

	1st T - PP	2nd T - PP	3rd T - PP	2nd T – 1st T	3rd T – 1st T	3rd T – 2nd T
Z	-1.960[b]	-1.542[b]	-.734[b]	-.514[b]	-.095[b]	-.369[b]
p-value	0.05	0.123	0.463	0.607	0.925	0.712

Results of the Wilcoxon Matched-Pairs Signed Rank Analysis among HP trimesters. **SD**, standard deviation, **1st T**, first trimester, **2nd T**, second trimester, **3rd T**, third trimester, **PP**, post-partum. a.Wilcoxon Signed Ranks Test b. Based on negative ranks

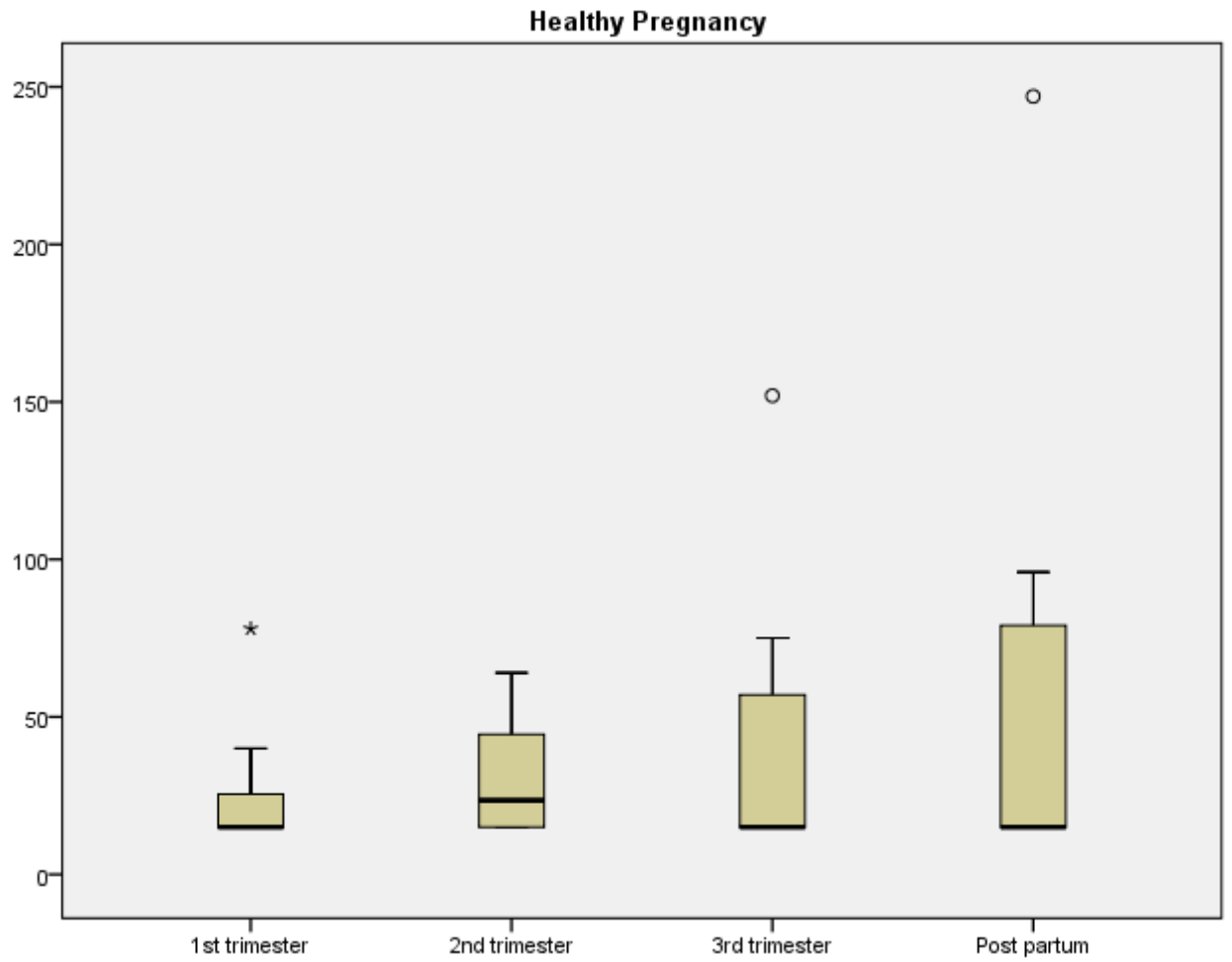


Figure 6.2 (a) Box plots depict distributions of measured calprotectin levels through pregnancy among health pregnant women. The asterisks and circles represent outliers.

6.5 Discussion

Most commonly measured laboratory tests change during healthy pregnancy, making it important to study FC in pregnancy and generate special reference values for this period (Larsson et al., 2008). In this study, we found FC to be elevated in IBD pregnancy patients and below the limit of detection in healthy pregnancy patients; thus FC is a useful biomarker in IBD during pregnancy.

The presence of FC in healthy patients is known. Costa et al. (2003) reported a median value of 11 µg/g in healthy, non-pregnant patients (n=34) (Costa et al., 2003). In our study, FC values in HP patients were shown to have a median value of 15 µg/g at each trimester and post-partum (n=46, 121 observations). To be noted, values in this study under the limit of detection (<30 µg/g) were recorded as 15 µg/g, thus, the value is likely to be lower.

Jost et al. (2014) presented FC values in pregnancy, with a mean of 18.8 ± 6.1 µg/g and no significant differences in levels throughout the perinatal (i.e. around the time of birth) period, as assessed by ELISA (n=7) (Jost, Lacroix, Braegger, & Chassard, 2014). The authors inferred these values may represent low-grade signs of intestinal inflammation, an observation shown elsewhere by increased pro-inflammatory cytokines in stool during the third trimester of pregnancy (Koren et al., 2012). Additionally, Urwin et al. (2014) observed a median FC concentration of 4.7 µg/g at in healthy pregnany controls at Week 38 gestation (Urwin et al., 2014). In this study, we calculated an overall mean FC value of 47 ± 61 µg/g (median 15 µg/g) (**Table 6.2**). Non-parametric analysis found no significant differences between assessment periods (**Table 6.4, 6.5**). In Jost et al. (2014), maternal feces was collected at four sampling points, 3–7 weeks prepartum and at days 3–6, 9–14, and 25–30 postpartum. In this study, longitudinal assessment of FC was performed throughout during each trimester (1-13 weeks, 14-26 weeks, 27-40 weeks) and within 1-6 months post-partum. Differences in the FC values between studies may be attributed to the differences in these sampling periods.

The concentration of FC in IBD pregnancy patients isn't well known. Median FC values were found to be elevated throughout T1-T3 and post-partum, 477, 337, 469 and 439 µg/g, respectively (**Table 6.4**). This indicates active organic inflammation in these patients, comparable to studies with other non-pregnant IBD patients (Costa et al., 2003). Non-parametric

analysis was not performed due to the post-partum sample size (n=1). These results show that FC is useful in detecting active inflammation in IBD pregnancy. Additionally, recruiting 17 total patients in this study group displays feasibility in this measurement.

The Mann-Whitney U test identified significant median differences in the FC values between the healthy and IBD pregnancy patients ($U=203.5$, $z=-8.474$, $p<0.001$). This finding demonstrates that unlike other biomarkers (CRP, ESR, etc), FC is not affected by pregnancy and can be used in this patient population.

Future work should calculate FC reference values based on fulfilling the recommendations of the International Federation of Clinical Chemistry's statistical treatment of reference values (Larsson et al., 2008). Specifically in IBD, future work should study the relationship between FC and pregnancy-related outcomes, such as pre-term birth, low birth weight, spontaneous abortion and mode of delivery. Also, the relative risk of adverse clinical outcomes amongst those with elevated FC compared with those without elevated levels should be calculated. Finally, since the gut microbiota is profoundly altered during pregnancy, future work should focus on the relationship between non-invasive biomarkers and gut microbiota (Koren et al., 2012).

6.6 Conclusion

This study established reference values for FC in healthy and IBD pregnancy, showing statistically significant differences between the groups, while touching on the feasibility of this analysis in this patient population.

Acknowledgements: Healthy pregnancy recruitment was conducted by Susan Kuling in the clinic of Drs. Martel and Mytopher from the Saskatoon Obstetrics and Gynecologic Consultants in Saskatoon, Saskatchewan. IBD pregnancy recruitment was conducted by Drs. Jones and Fowler from the MDIBDC at the University of Saskatchewan.

CHAPTER 7

FECAL MICROBIOTA AND SHORT CHAIN FATTY ACIDS IN NEWLY DIAGNOSED IBD PATIENTS

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Not yet published

The relationship between mucosa and resident microbial flora ('microbiota') heavily influences human health. Gut bacteria play an integral role in health by exerting protective, structural and metabolic effects. A change in the composition of the gut bacteria, or "dysbiosis" is hypothesized to play a role in the development of IBD. The majority of gut bacteria belong to two phyla, *Bacteroidetes* and *Firmicutes.* A reduced concentration of *Bacteroidetes* and *Firmicutes*, responsible for the anaerobic production of short chain fatty acids (SCFA) (i.e. acetate, propionate, and butyrate) integral to human health, is well documented in IBD. Specifically, butyrate has many anti-inflammatory and anti-proliferative properties, and is decreased in IBD, making it an attractive marker of study. It is unknown how SCFA are influenced by IBD disease activity and severity, therapy and disease type. Furthermore, this study only included newly diagnosed IBD patients, to limit the confounding that plagues most observational studies examining patients with longstanding disease. This study examined the relationship between gut microbiota, SCFA and biomarkers in IBD.

Author role: I completed the SCFA lab analysis, statistical analysis and manuscript preparation and writing for this chapter.

Fecal Microbiota and Short Chain Fatty Acids in Newly Diagnosed IBD Patients

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Keywords: Short chain fatty acids, Inflammatory Bowel Disease, Diagnosis, Biomarkers

7.1 Introduction

The pathogenesis and etiology of IBD, although not completely understood, is hypothesized to develop as a result of the interaction between three factors - genetic susceptibility, environmental exposures and host immune response (Cashman & Shanahan, 2003). The intestinal microflora is a key environmental factor influencing IBD in both subtypes, CD and UC, since genetically engineered animal models of IBD do not develop colitis under germ-free conditions (i.e. knock-out mice without gut bacteria) (Taurog et al., 1994).

Human health is highly dependent on the relationship between human mucosal sites and the resident microbial flora ('microbiota') (Vinolo, Rodrigues, Nachbar, & Curi, 2011). Healthy individuals have a microbial ecology that is characterized by a stable dominant microbiota, exhibiting high biodiversity and resilience (Marteau, 2009). In contrast, the microbiota in IBD patients is less diverse (Kelly & Mulder, 2012). Reduced diversity may result in innappropriate mucosal cellular responses, that could cause the prolonged inflammatory response observed in intestinal disease (Murphy et al., 2012). A difference in the composition of the gut bacteria, or "dysbiosis", has been observed between IBD patients and healthy controls, although the significance to IBD pathogenesis is unknown (Froyland, 2010; Swidsinski et al., 2002). If this imbalance could be recognized it could serve as an important diagnostic tool in IBD (Froyland, 2010).

The role of the gut microbiota in IBD is discussed in **Chapter 2.3.1.2** of this document, along with being recently reviewed elsewhere (Hold et al., 2014). Microbiota can be organized by taxonomic rank – from phylum to species, through class, order, family and genus (Froyland, 2010). The major phyla found within human GI tracts, regardless of health or disease, are found within four bacterial categories: the *Firmicutes*, *Bacteriodetes*, *Proteobacteria* and *Actinobacteria* (Eckburg et al., 2005; Frank et al., 2007; Ley, Turnbaugh, Klein, & Gordon, 2006). Most of the microbiota in healthy humans belong to the *Bacteroidetes* and *Firmicutes* phyla (Eckburg et al., 2005; Hold, Pryde, Russell, Furrie, & Flint, 2002; Suau et al., 1999). Higher levels of *Bacteroidetes* in CD patients compared with controls have been observed (Keighley et al., 1978; Ruseler-van Embden & Both-Patoir, 1983). Furthermore, a reduced abundance of the phyla *Firmicutes* has been well documented in IBD patients (Frank et al., 2007; Peterson, Frank, Pace, & Gordon, 2008; Sokol et al., 2006). Within *Firmicutes*, the species, *Clostridium leptum*

comprises up to 25% of the fecal microbiota population (Lay et al., 2005; Sghir et al., 2000). This group is largely responsible for the fermentation of unabsorbed carbohydrate, producing short-chain fatty acids, and has been shown to be reduced in both CD and UC (Kabeerdoss, Sankaran, Pugazhendhi, & Ramakrishna, 2013). The most abundant member of *C. Leptum* is *Faecalibacterium prausnitzii* a major producer of butyrate in the gut (Louis & Flint, 2009; Sokol et al., 2008). The microbial composition of the gut during the development and diagnosis of IBD is not well known, making the study of newly diagnosed populations important

SCFA are the major metabolic products of anaerobic fermentation of dietary compounds in the colon by bacteria (Treem et al., 1994). The amount of SCFA produced are dependent on the number and type of microbiota, availability of substrate and gut transit time (Argenzio & Southworth, 1975; Cook & Sellin, 1998; Owens & Isaacson, 1977; Roberfroid et al., 2010; Wong, de Souza, Kendall, Emam, & Jenkins, 2006). Butyrate exerts many positive health effects, playing a key role in the physiology and health of the colonic mucosa (Wong et al., 2006). Additionally, butyrate metabolism is impaired in IBD, due to a decrease in β-oxidation (Roediger, 1980; Thibault et al., 2010). Thus, butyrate is suggested to be a factor in the development and treatment of IBD, mainly CD and UC (Cummings, 1997; Di Sabatino et al., 2005). In the majority of SCFA studies in human IBD, fecal concentrations of SCFA are positively correlated with clinical measures of disease activity (Mortensen & Clausen, 1996; Roediger et al., 1982; Treem et al., 1994). However, the concentration of organic acids in relation to disease activity, in both active and inactive states in IBD, have been inconsistent (Hove & Mortensen, 1995; Hove et al., 1994; Huda-Faujan et al., 2010). Little is known about the relationship between the standard biomarkers assessed in IBD, serum marker hsCRP, the fecal biomarkers, FC and Lf, and SCFA and microbial populations in the gut, particularly the butyrate producing *F. prausnitzii.*

-

7.2 Objectives

The aim of this study was to observe the fecal concentrations of SCFA and microbiota in newly diagnosed IBD patients, assess how they differ between disease type and severity, and relate to objective markers of inflammation.

7.3 Materials and methods

Patient recruitment: Eligible subjects were diagnosed with IBD within the past 12 months and seen at the MDIBDC. Individuals ages 18 and older with a diagnosis of IBD based on standard criteria and able to provide written informed consent were eligible to participate in the study conducted from November 2012 to July 2014 (see section **5.3**) (Lennard-Jones, 1989). Exclusion criteria in this cohort consisted of patients with infectious diseases, end-stage renal, cardiovascular or hepatic disease or who were pregnant. This study was approved by the University of Saskatchewan's Biomedical Research Ethics Board (Bio-REB 09-26).

Experimental design: Patient demographics (age, gender, disease type, location, medication therapy, smoking status) were collected from all participants. At baseline and follow-up (3 – 6 months), detailed endoscopic (Simple Endoscopic Score – CD [SES-CD], Mayo Score for UC) and clinical disease activity measures (CD Activity Index [CDAI], partial Mayo score for UC) were calculated. At baseline and follow-up (3 – 6 months), blood and fecal samples were collected for biomarker (FC, Lf, hsCRP), microbiota and SCFA determination.

Blood was drawn by trained Royal University Hospital personnel and used for hsCRP determination. Fecal samples (two 50-gram aliquots) were collected in sterile containers, one left thawed for immediate biomarker determination, and one frozen immediately and delivered to the Mary Irwin Laboratory of Nutrition at the University of Saskatchewan for microbiota and SCFA analysis. Samples were stored at -80 °C until analysis.

Disease Activity: Endoscopic disease activity was scored by colonoscopists using the SES-CD and Mayo Score for UC. SES-CD was categorized as active >7, inactive ≤7, and Mayo score active >6 points, inactive ≤6. Clinical disease activity was scored using the CDAI for CD and partial Mayo score for UC. CDAI was categorized as active ≥ 150, inactive < 150, and Partial Mayo scores active > 2, inactive ≤2.

Biomarker analysis: hsCRP was tested using the high sensitivity CRP (latex) reagent on the c501 analyzer (Roche Diagnostics Canada, Laval, QC, CA) using the manufacturer's reagents and calibrators (Sánchez et al., 2002). Results were calculated in milligrams per millilitre (mg/mL).

FC was determined using a Quantum Blue® FC High Range Rapid Test using the Quantum Blue Reader® point-of-care (POC) desk-top reader (ALPCO Immunoassays, Salem, NH, USA). Approximately 30 mg of fecal sample was pressed into a base cap and fitted on the extraction tube. This device was filled with 4 mL of extraction buffer and homogenized by vortex for 1 minute. A 1:16 dilution was performed with chase buffer and 80 µL was positioned into the test cartridge for analysis. Each run took 15 minutes to complete and results were displayed in micrograms per gram (µg/g) on the POC display.

Lf was determined using IBD-SCAN® (TechLab, Inc, Blacksburg, VA, USA). Approximately 450 mg of fecal sample was weighed in an Eppendorf tube and diluted at 1:100 to 1:10,000. Test samples, standards and quality controls were plated in a 96-well plate provided with the kit. The plate was read at 450/620 nanometres wavelength microplate reader and results were calculated in micrograms per millilitre (µg/mL).

Microbiota analysis: The frozen fecal sample was thawed and brought to Contango Strategies (Saskatoon, SK) immediately for analysis. Approximately 1 gram of the fecal sample was separated, homogenized and used for analysis. Targeted DNA sequencing was used to identify bacteria present in each sample via polymerase chain reaction (PCR) amplification of the v3/v4 region of the 16S ribosomal RNA gene (Klindworth et al., 2013) Library preparation and sequencing was performed as per the manufacturer's instructions for MiSeq v3 paired-end 300 bp sequencing (Illumina, San Diego, CA, USA). Library preparation included positive and negative controls, with the former consisting of mock communities, and the latter where no DNA is added to the PCR, and the sample is carried through to sequencing. After sequencing, the forward and reverse reads were merged using PANDAseq (Masella, Bartram, Truszkowski, Brown, & Neufeld, 2012). All sequences were then filtered and reads were considered to be low quality and discarded if they did not meet the following criteria: average quality greater than Q30, longer than 350 bp, and exact match to the forward primer. Additionally, if the read had

any base called as N (unknown) it was discarded. The forward and reverse primers were then removed from each sequence. Bioinformatics pipelines consisting of internally developed scripts and selected QIIME scripts (Caporaso et al., 2010; Edgar, 2010) were used to process the reads. Similar sequences were clustered into groups called Operational Taxonomic Units (OTUs) using a 97% identity threshold and the *pick_de_novo_otus.py* script. All OTUs with less than 10 representative sequences across all samples were discarded as a quality filtering step to remove OTUs that may have arisen due to sequencing errors. Taxonomic classification of the OTUs were performed using the Greengenes database version 13_8 (DeSantis et al., 2006; McDonald et al., 2012). Microbial diversity was determined through observed species, Simpson reciprocal, and the Shannon Index, measures of microbial diversity (Magurran, 1988).

SCFA analysis: Phosphoric acid, 85%, and hydrochloric acid, acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocaproic acid (internal standard) an caproic acid, all purchased at 99%, were obtained from Sigma-Aldrich (Sigma-Aldrich Canada Co., Oakville, ONT, CA) (**Table 7.1**). CHROMASOLV HPLC grade water was also purchased from Sigma-Aldrich. For GC analysis, the internal standard, isocaproic acid (ICA), was prepared by adding 300 µL of ICA to 20 mL of 25% phosphoric acid, 20 mL of water, and bringing up to volume in a 100 mL volumetric flask. The mixed standard was prepared by adding the individual fatty acids of interest (**Table. 7.1**), with 20 mL of 25% phosphoric acid, brought up to volume in a 100 mL volumetric flask. Weight of the added internal standard and fatty acids of interest were recorded for determination of final concentration.

From the remaining thawed fecal sample (from the microbiota analysis), between 1-5 grams was homogenized, weighed and placed in a 30mL Nalgene bottle (in duplicate). A similar amount of 2N HCL and 1 mL of 25% m-phosphoric acid was added to the tube and vortexed. The sample was centrifuged for 20 minutes at 20,000 x g. Supernatant was filtered through a 25 mm 0.45 µM Millex-HPF syringe filter (Millipore, Etobicoke, ONT, CA). The filtered sample was transferred to four Eppendorf tubes and centrifuged at 22,000 x g for 10 minutes. Samples were then ready to be analyzed. One millilitre (1 mL) of filtered supernatant was transferred to GC vials, along with 0.2 mL of internal standard for analysis.

Table 7.1 Short chain fatty acids of interest in mixed standard

Fatty acid	Volume (µL)	Molar mass (g/mol)
Acetic acid (AA)	300	60.05
Propionic acid (PA)	200	74.08
Isobutyric acid (IBA)	50	88.11
Butyric acid (BA)	100	88.11
Isovaleric acid (IVA)	50	102.13
Valeric acid (VA)	50	102.13
Caproic acid (CA)	50	116.20

The GC-FID system consisted of an Agilent 6890 GC system equipped with a flame ionization detector (FID) and an automatic liquid sampler (Agilent Technologies, Palo Alto, CA, USA). A GC – FID chromatogram with a mixed SCFA standard is shown in **Figure 7.1**. The column was a ZB-FFAP capillary column (Phenomoenex, Torrance, CA, USA) (30.0 m × 320 µm × 0.25 µm). Helium was used as a carrier gas at 1.2 mL/min. A 1 µL sample injection was completed in split mode, with an initial temperature of 170°C. GC grade methanol (wash) and acetonitrile were used as solvents. A wash and standard injection was made after every 10 sample injections. Data acquisition was completed with Chemstation (Hewlett– Packard, Palo Alto, CA, USA). Quantitative analysis was performed using relative response factors for the short chain fatty acids determined with an internal standard, isocaproic acid (ICA) (**Appendix B**). Additionally, samples were freeze-dried using a FreeZonePlus 6L Dry System to analyze water content for dry weight calculations (Labconco, Kansas City, MO, USA). Full standard operating procedure for this analysis can be found in **Appendix B**.

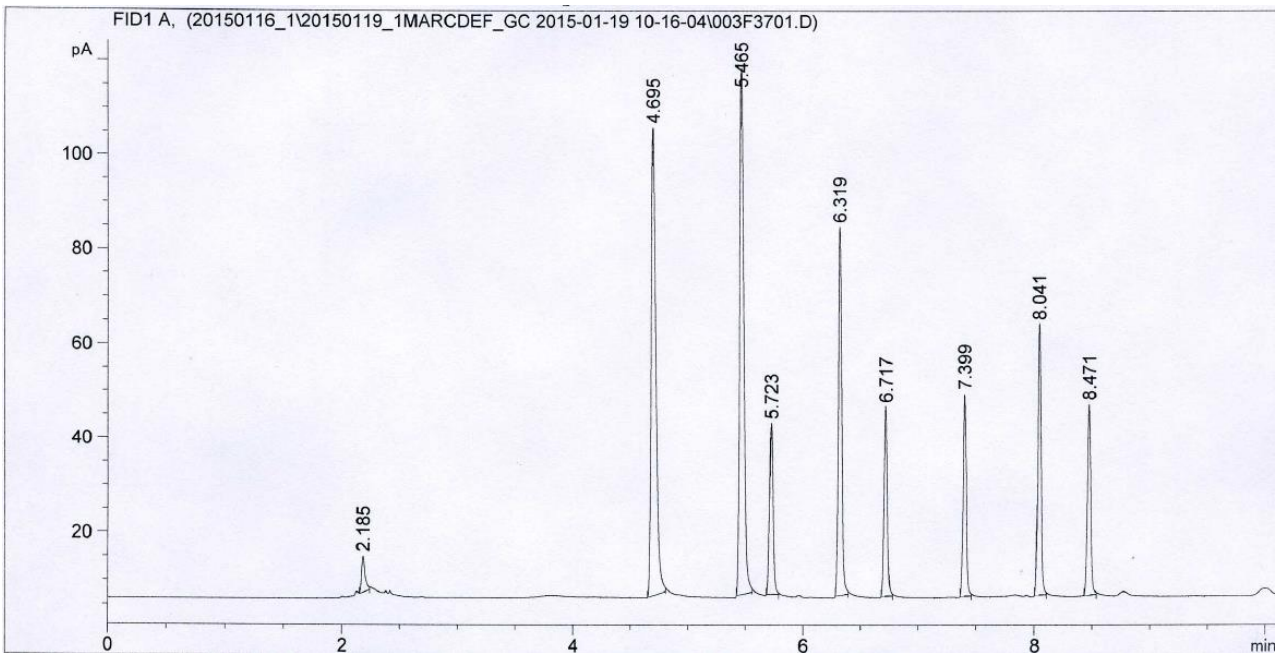

Figure 7.1 Gas chromatography - flame ionization chromatogram of SCFA mixed standard displaying short chain fatty acid peaks. SCFA shown in order of elution: Acetic Acid (4.695 min), Propionic Acid (5.465 min), Isobutyric Acid (5.723 min), Butyric Acid (6.319 min), Isovaleric Acid (6.717 min), Valeric Acid (7.399 min), Isocaproic Acid (8.041 min), Caproic Acid (8.471 min).

Statistics: Descriptive statistics were used to describe patient demographics and biomarker concentrations. Data are presented as mean ± standard deviation unless otherwise noted**.** Biomarkers are presented as median values with interquartile ranges (not normally distributed). Unpaired t-test was used to compare means in different disease states (CD vs. UC). Paired t-test was used to compare means at different time points (Baseline and Follow-up). The pooled IBD patients were split into quartiles based on *F. prausnitzii* percent relative abundance (n=6). A one-way ANOVA was used to compare means between quartiles of the *F. prausnitzii* group. Homogeneity of variances was assessed using the Levene statistic. Tukey post hoc analysis was used to reveal statistically significant differences between groups. A one-way ANOVA was used to determine the relationship between SCFA, typical IBD biomarkers and markers of microbial diversity. Pearson correlation was used to assess relationship between biomarkers and microbiota and SCFA.

7.4 Results

During recruitment, 25 patients were screened for potential enrolment and 15 participants who met the inclusion and exclusion criteria were subsequently enrolled. Nine of these patients submitted follow-up fecal samples for a total of 24 fecal samples collected (15 baseline, 9 follow-up, 24 total). The cohort was split between 27% male and 73% female, with a mean age of 33.8 years (range 20 - 60 years). In this study, 73% of patients reported never smoking, 13% current smokers, and 13% did not answer. The ethnicity of the patient population was 93% Caucasian and 7% Aboriginal. Eleven participants (73%) were newly diagnosed with CD and the other four (27%) had UC. The demographic and clinical characteristics of this cohort are shown in **Table 7.2**. Of the 11 CD patients, 64% had ileocolonic disease. Disease location was evenly split in UC, 50% of patients had pancolonic disease and 50% had procosigmoditis.

hsCRP, FC and Lf were elevated and outside of the normal range at enrolment. At enrolment, the median hsCRP concentration was 8.0 mg/L (normal 0 – 7.0 mg/L), FC concentration was 1236 μg/g (normal <50 μg/g), and fecal Lf concentration was 53 μg /mL (normal 0 - 7.24 μg/mL) (**Table 7.3**).

Table 7.2 Demographics and clinical characteristics of the newly diagnosed cohort (n=15)

Patient	Age	Gender	Disease location	Smoking	Therapy
CD1	52	M	ileocolonic	no	anti-TNF agent
CD2	24	F	ileocolonic	no	immunomodulator
CD3	20	F	ileocolonic	no	anti-TNF agent
CD4	25	M	ileocolonic	no	immunomodulator
CD5	22	F	ileocolonic	no	anti-TNF agent
CD6	34	F	ileocolonic	no	immunomodulator
CD7	60	F	colonic	no	none
CD8	28	F	colonic	yes	immunomodulator
CD9	32	F	colonic	yes	immunomodulator
CD10	52	F	ileal	no	immunomodulator
CD11	32	F	ileocolonic	n/a	ASA
CD	**34.6 ± 13.7**	**2M / 9F**			
UC1	20	M	pancolonic	n/a	ASA
UC2	34	F	proctosigmoidal	no	ASA
UC3	35	F	proctosigmoidal	no	ASA
UC4	37	M	pancolonic	no	ASA
UC	**31.5 ± 7.8**	**2M / 2F**			
Total	**33.8 ± 12.2**	**4M / 11F**			

Baseline demographics in 15 newly diagnosed IBD patients (11 Crohn's disease, 4 Ulcerative colitis). Disease location is confirmed by colonoscopy. **ASA,** amino-salicylic acid, **Anti-TNF agent,** anti-tumour necrosis factor agent**, CD**, Crohn's disease, **UC**, ulcerative colitis.

Table 7.3 Biomarker concentration in a full cohort of newly diagnosed patients (n=15)

Biomarker	Median	IQR	Q1 - Q3	Normal Range
hsCRP (mg/L)	8.0	21.5	3.0 - 24.5	0 - 7.0
FC (µg/g)	1236	1481	580 - 1772	< 50
Lf (µg/mL)	53.0	279	6.0 - 284.5	0 - 7.24

Results of biomarker concentration of in full cohort of 15 IBD patients. Results are expressed as median values. **IQR**, Interquartile range, **Q1**, quartile 1, **Q3**, quartile 3, **hsCRP,** high sensitivity C-reactive protein, **FC,** calprotectin, **Lf**, lactoferrin.

Quantifiable DNA for microbiota analysis was obtained from all 15 baseline and 9 follow-up samples. 16s library sequencing yielded an average of 164,770 reads per sample. The negative control (i.e., no DNA added) generated minimal reads, with 616 reads and only ~3% retained after quality filtering. Positive mock community control samples passed internal QC standards.

Firmicutes were higher in CD (74.4% ± 14.3) than UC (64.2% ± 21.6), while *Bacteroidetes* was lower in CD (15.1% ± 13.4) than UC (21.4% ± 22.9) (**Table 7.4**). *Bacteroidetes* were lower at follow-up (8.9% ± 8.3) than baseline (16.8% ± 15.8), while *Firmicutes* were increased at follow-up (77.8% ± 10.2) than baseline (71.7% ± 16.4) (**Figure 7.2**).

The major phyla *Firmicutes* and *Bacteroidetes* were reduced in active disease compared with inactive disease, with *Actinobacteria*, *Proteobacteria*, and *Fusobacteria* increased (**Figure 7.3**). The concentration of all major SCFA in this study was also shown to be higher in active vs. inactive IBD patients (**Figure 7.4**). Relative abundance of microbiota between paired samples is shown in **Figure 7.5**

The concentration of SCFA increased across the *F. prausnitzii* quartiles. Acetic, propionic, and butyric acid (AA, PA, BA), the three primary SCFA respectively, increased across quartiles (**Table 7.5**). Generally, biomarkers decreased across *F. prausnitzii* quartiles. High-sensitivity C-reactive protein decreased from the 1st quartile to the 2nd, increased in the 3rd, but finally decreased in the 4th quartile. A similar trend was observed in the fecal biomarkers, FC and Lf, as the markers decreased from the 1st quartile to the 2nd, increased in the 3rd, and decreased in the 4th quartile but not below the values found in the 2nd. Microbial diversity increased across *F. prausnitzii* quartiles. The number of observed species and Shannon Index,

increased from the 1st quartile to the 2nd, decreased in the 3rd, and increased again in the final quartile

The assumption of homogeneity of variances was violated for FC and hsCRP ($p < 0.05$). There were no statistically significant differences between quartiles of *F. prausnitzii* for AA, PA, Lf, FC and hsCRP. However, statistically significant differences were observed between quartiles of *F. prausnitzii* and observed species ($p < 0.001$, between 1st and 4th), Shannon Index ($p < 0.001$, 1st and 2nd), and BA ($p < 0.05$, 1st and 4th).

Of the major fecal SCFA (measured in µmol/g wet feces), AA was higher in CD than UC (31.5 ± 9.2 vs. 26.2 ± 13.0) and reduced at follow-up compared with baseline (30.1 ± 10.1 vs. 27.6 ± 13.1). PA was lower in CD than UC (7.5 ± 3.9 vs. 9.7 ± 11.4) and reduced at follow-up compared with baseline (8.1 ± 6.3 vs. 6.1 ± 4.3). BA was lower in CD than UC (6.2 ± 3.2 vs. 6.8 ± 7.0) and reduced at follow-up compared with baseline (6.4 ± 4.2 vs. 6.3 ± 5.1). When compared with healthy control values (Nilsson, Johansson, Nilsson, Björck, & Nyman, 2008) the difference in SCFA were statistically significant ($p<0.001$).

Proteobacteria was correlated with all biomarkers, Lf and FC ($p>0.01$) and hsCRP ($p>0.05$). Propionic acid correlated with Lf ($p>0.05$) (**Table 7.7**).

Table 7.4 Abundance of fecal microbiota in newly diagnosed IBD patients

Phyla	CD (n=11)	UC (n=4)	Baseline (n=15)	Follow-up (n=9)	All (n=24)	Healthy (n=20)[1]	Healthy (n=5)[2]
Actinobacteria	3.6 ± 2.2 (1.1 – 7.7)	4.1 ± 3.7 (0.1 – 7.5)	3.7 ± 2.5 (0.1 – 7.7)	8.4 ± 10.2 (1.5 – 34.4)	5.5 ± 6.7 (0.1 – 34.7)		0.5 ± 0.4 (nd – 1.2)[2]
Bacteroidetes	15.1 ± 13.4 (nd – 33.5)	21.4 ± 22.9 (nd – 46.6)	16.8 ± 15.8 (nd – 46.6)	8.9 ± 8.3 (nd – 18.8)	13.8 ± 13.8 (nd – 46.6)	53.9[1]	31.7 ± 14.9 (12.7 – 48.0)[2]
Firmicutes	74.4 ± 14.3 (52.8 – 91.1)	64.2 ± 21.6 (38.8 – 87.9)	71.7 ± 16.4 (38.8 – 91.1)	77.8 ± 10.2 (61.9 – 91.0)	74.0 ± 14.4 (38.8 – 91.1)	38.9[1]	63.4 ± 13.3 (50.8 – 82.4)[2]
Fusobacteria	2.6 ± 7.5 (nd – 25.0)	nd ± 0.1 (nd – 0.2)	1.9 ± 6.4 (nd – 25.0)	nd ± 0.0 (nd – 0.1)	1.2 ± 5.1 (nd – 25.0)	0.5[1]	0.2 ± 0.5 (nd – 1.2)[2]
Proteobacteria	3.6 ± 5.0 (nd – 14.8)	8.1 ± 11.2 (0.1 – 24.2)	4.8 ± 7.0 (nd – 24.2)	3.4 ± 5.6 (0.1 – 17.5)	4.2 ± 6.4 (nd – 24.2)		3.9 ± 3.6 (0.8 – 9.9)[2]
Tenericutes	0.1 ± 0.5 (nd – 1.5)	nd ± 0.0 (nd – 0.1)	0.1 ± 0.4 (nd – 1.5)	nd ± 0.0 (nd)	0.1 ± 0.3 (nd – 1.5)		
Verrucomicrobia	0.2 ± 0.7 (nd – 2.3)	1.5 ± 3.0 (nd – 5.9)	0.6 ± 1.6 (nd – 5.9)	1.3 ± 3.5 (nd – 10.6)	0.8 ± 2.4 (nd – 10.6)		0.2 ± 0.3 (nd – 0.7)[2]
Other	0.4 ± 0.2 (0.2 – 0.6)	0.3 ± 0.1 (0.2 –0.5)	0.4 ± 0.2 (0.2 – 0.6)	0.2 ± 0.1 (nd – 0.4)	0.3 ± 0.2 (nd – 0.6)		
Shannon Index	5.1 ± 0.8 (3.7 – 6.02)	4.7 ± 1.8 (2.1 – 6.4)	5.0 ± 1.1 (2.1 – 6.4)	5.0 ± 0.7 (3.9 – 6.1)	5.0 ± 0.9 (2.1 – 6.4)	5.1[1]	3.6 ± 0.2 (3.5-3.9)[2]

Results showing the relative abundance of fecal microbiota in newly diagnosed IBD patients, expressed as relative percentage (%) of bacteria in feces (mean ± SD [range]). Analysis found minimal to negligible cyanobacteria, euryarchaeota or TM7 bacteria present. Microbiota diversity was expressed by the Shannon Index. Shown graphically in Appendix D. **nd**, no detection; **CD**, Crohn's disease; **UC**, ulcerative colitis, **Baseline**, pooled baseline, **Follow-up,** pooled follow-up. [1](Wu et al., 2013) – fecal samples, [2](Walker et al., 2011) - biopsies.

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Table 7.5 Quartiles of *Faecalibacterium prausnitzii* in pooled IBD patients (total, n = 24; quartile, n = 6)

	1st (low)	**2nd**	**3rd**	**4th (high)**	**TOTAL**	**p-value**
Obs. Species	242.7 (85.1)**	610.2 (226.4)	543.5 (171.6)	661.0 (62.1)**	514.3 (217.8)	<0.001
Shannon Index	3.8 (0.9)**	5.5 (0.8)**	5.1 (0.5)	5.5 (0.3)	5.0 (0.9)	<0.001
AA	24.9 (11.3)	27.5 (14.9)	27.6 (8.0)	36.6 (7.7)	29.2 (11.1)	0.293
PA	4.0 (4.3)	7.2 (6.0)	7.5 (3.0)	10.6 (7.5)	7.3 (5.6)	0.260
BA	3.1 (2.8)*	5.9 (5.9)	6.1 (2.2)	10.2 (3.6)*	6.3 (4.5)	0.040
FC (µg/g)	3862 (3855)	910 (514)	1107 (1276)	1040 (795)	1730 (2316)	0.066
Lf (µg/ml)	275.6 (278.7)	64.1 (105.5)	148.7 (136.7)	98.6 (153.6)	146.7 (187.7)	0.227
hsCRP (µg/L)	26.0 (26.8)	11.5 (11.9)	17.3 (32.7)	6.2 (6.4)	15.3 (22.0)	0.469

Table showing trends of short chain fatty acids (SCFA), microbial diversity and biomarkers across *F. prausnitzii* quartiles. SCFA results are expressed in µmol/g wet feces (mean [SD]). **AA**, acetic acid, **PA,** propionic acid; **BA,** butyric acid; **FC,** calprotectin; **Lf,** lactoferrin; **CD**, Crohn's disease. Microbiota diversity was expressed by total observed species and the Shannon Index. A one-way ANOVA analysis was used to compare means between groups. **significance $p < 0.001$, *significance $p < 0.05$ between groups.

Table 7.6 Fecal SCFA concentration in newly diagnosed IBD patients

SCFA	CD (n=11)	UC (n=4)	Baseline (n=15)	Follow-up (n=9)	All (n=24)	Healthy (n=20)[1]	p-value
AA	31.5 ± 9.2 (17.6 –	26.2 ± 13.0 (12.5 – 40.4)	30.1 ± 10.1 (12.5 – 44.8)	27.6 ± 13.1 (7.2 – 48.2)	29.2 ± 11.1 (7.2 – 48.2)	54.2 ± 2.5 (19.5 -126.2)	<0.0001
PA	7.5 ± 3.9 (1.8 – 15.6)	9.7 ± 11.4 (nd – 25.3)	8.1 ± 6.3 (nd – 25.3)	6.1 ± 4.3 (0.2 – 12.6)	7.3 ± 5.6 (nd – 25.3)	11.6 ± 0.5 (4.5 -22.5)	<0.001
IBA	0.7 ± 0.4 (nd – 1.5)	0.8 ± 0.6 (nd – 1.3)	0.7 ± 0.4 (nd -1.5)	0.3 ± 0.3 (nd – 1.0)	0.6 ± 0.4 (nd – 1.5)	2.1 ± 0.1 (4.1 -38)	<0.0001
BA	6.2 ± 3.2 (1.7 – 11.7)	6.8 ± 7.0 (nd – 15.3)	6.4 ± 4.2 (nd – 15.3)	6.3 ± 5.1 (0.3 – 16.0)	6.3 ± 4.5 (0.0 – 16.0)	13.9 ± 0.8 (0.9 - 4.8)	<0.0001
IVA	1.0 ± 0.6 (nd – 1.9)	1.0 ± 0.7 (nd – 1.7)	1.0 ± 0.6 (nd – 1.9)	0.4 ± 0.4 (nd – 1.2)	0.8 ± 0.6 (nd – 1.9)	1.5 ± 0.1 (0.2 – 4.1)	<0.0001
VA	0.6 ± 0.5 (nd – 1.2)	0.9 ± 0.6 (nd – 1.4)	0.7 ± 0.5 (nd – 1.4)	0.8 ± 0.8 (nd – 2.3)	0.7 ± 0.6 (nd – 2.3)	2.2 ± 0.2 (0.3 – 14)	<0.0001
CA	0.1 ± 0.2 (nd – 0.8)	0.2 ± 0.2 (nd – 0.4)	0.1 ± 0.2 (nd – 0.8)	nd (nd – 0.1)	0.1 ± 0.2 (nd – 0.8)	1.1 ± 0.1 (0.1 – 6.3)	<0.0001
Molar ratio (aa:pa:ba)	**70:16:14**	**61:23:16**	**68:18:14**	**69:15:16**	**68:17:15**	**68:15:17**	

Results are expressed in µmol/g wet feces (mean ± SD [Range]). **nd**, no detection; **CD**, Crohn's disease; **UC**, ulcerative colitis, **AA**, acetic acid, **PA,** propionic acid, **IBA,** isobutyric acid, **BA,** butyric acid, **IVA**, isovaleric acid**, VA**, valeric acid, **CA**, caproic acid, **Baseline**, pooled baseline, **Follow-up,** pooled follow-up. [1](U. Nilsson et al., 2008) Results are expressed in µmol/g wet feces (Mean ± SEM [Range]). An unpaired t-test was used to compare IBD (n=24) and healthy[1] values.

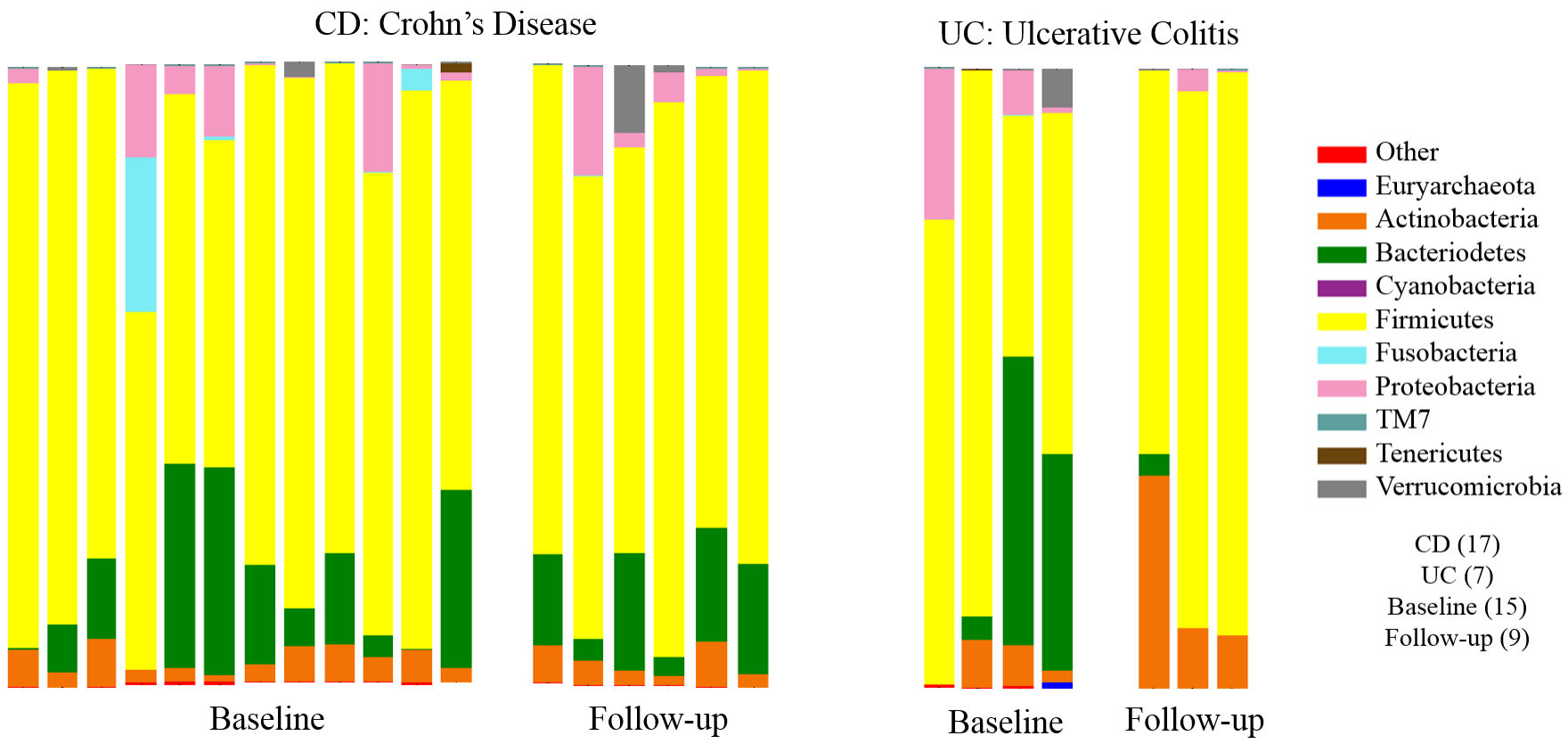

Figure 7.2 Relative abundance of bacterial phyla in 24 human fecal samples from IBD patients. Phyla legend found on right. Data is expressed as percent (%) composition.

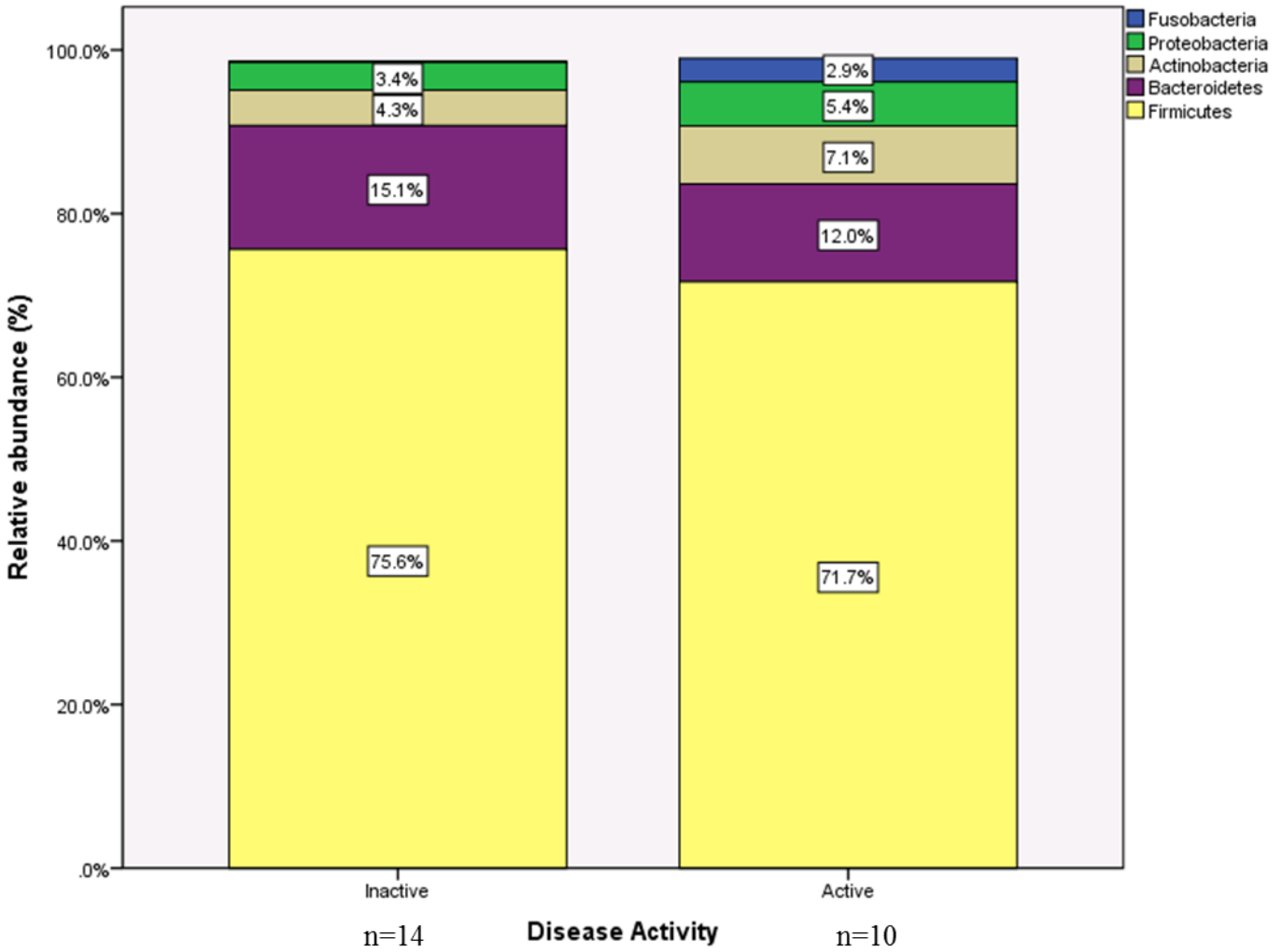

Figure 7.3 Relative abundance of bacterial phyla between inactive and active IBD patients (n=24). Groups were categorized by Crohn's disease Activity Scores (active ≥ 150, inactive < 150) for Crohn's disease and Partial Mayo scores (active > 2, inactive ≤2) Legend found on right. Data is expressed as relative abundance (%)

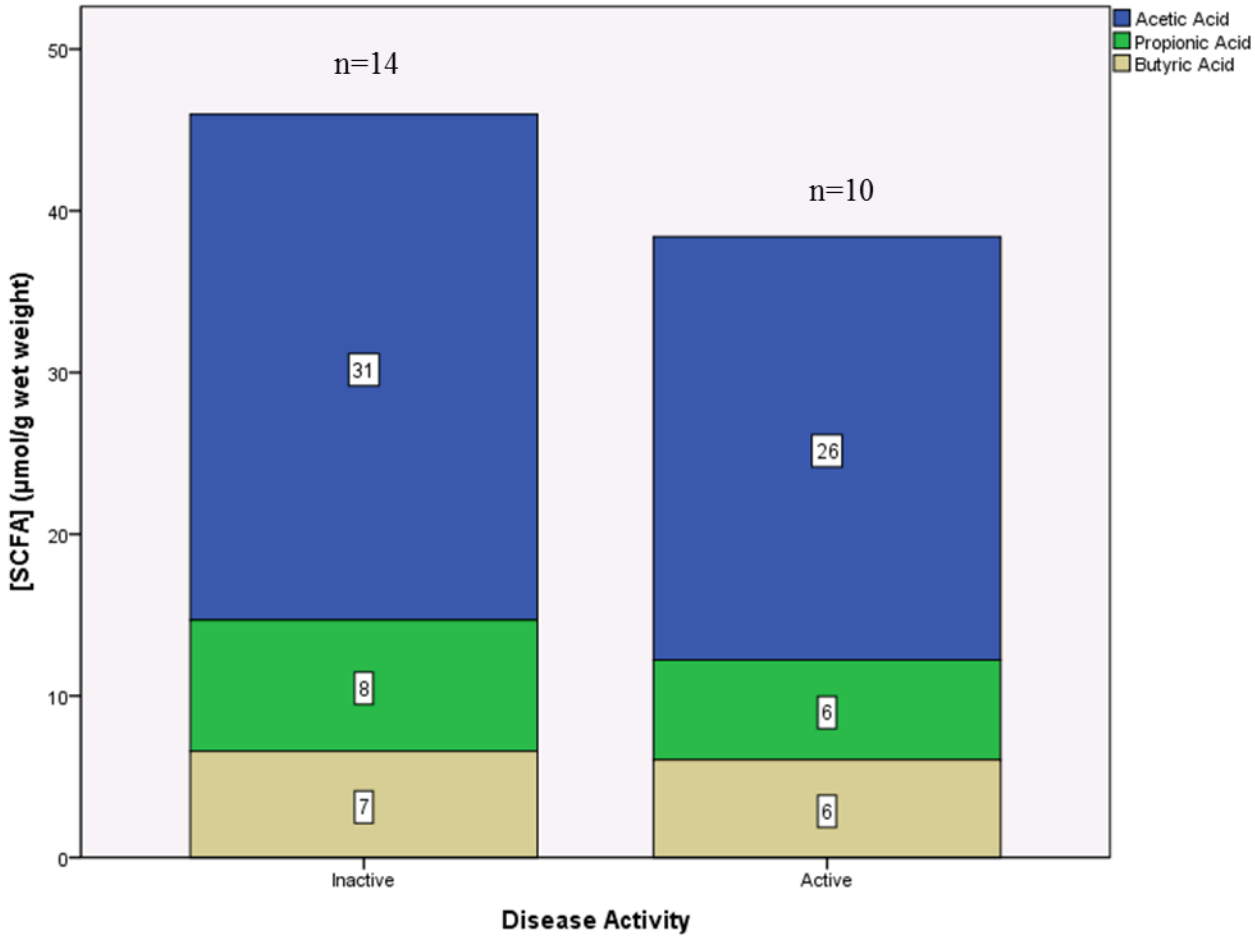

Figure 7.4 Concentration of fecal short chain fatty acids between inactive and active IBD patients (n=24). Groups were categorized by Crohn's disease Activity Scores (active ≥ 150, inactive < 150) for Crohn's disease and Partial Mayo scores (active > 2, inactive ≤2) Legend found on right. Data is expressed as micromole per gram of wet feces (µmol/g)

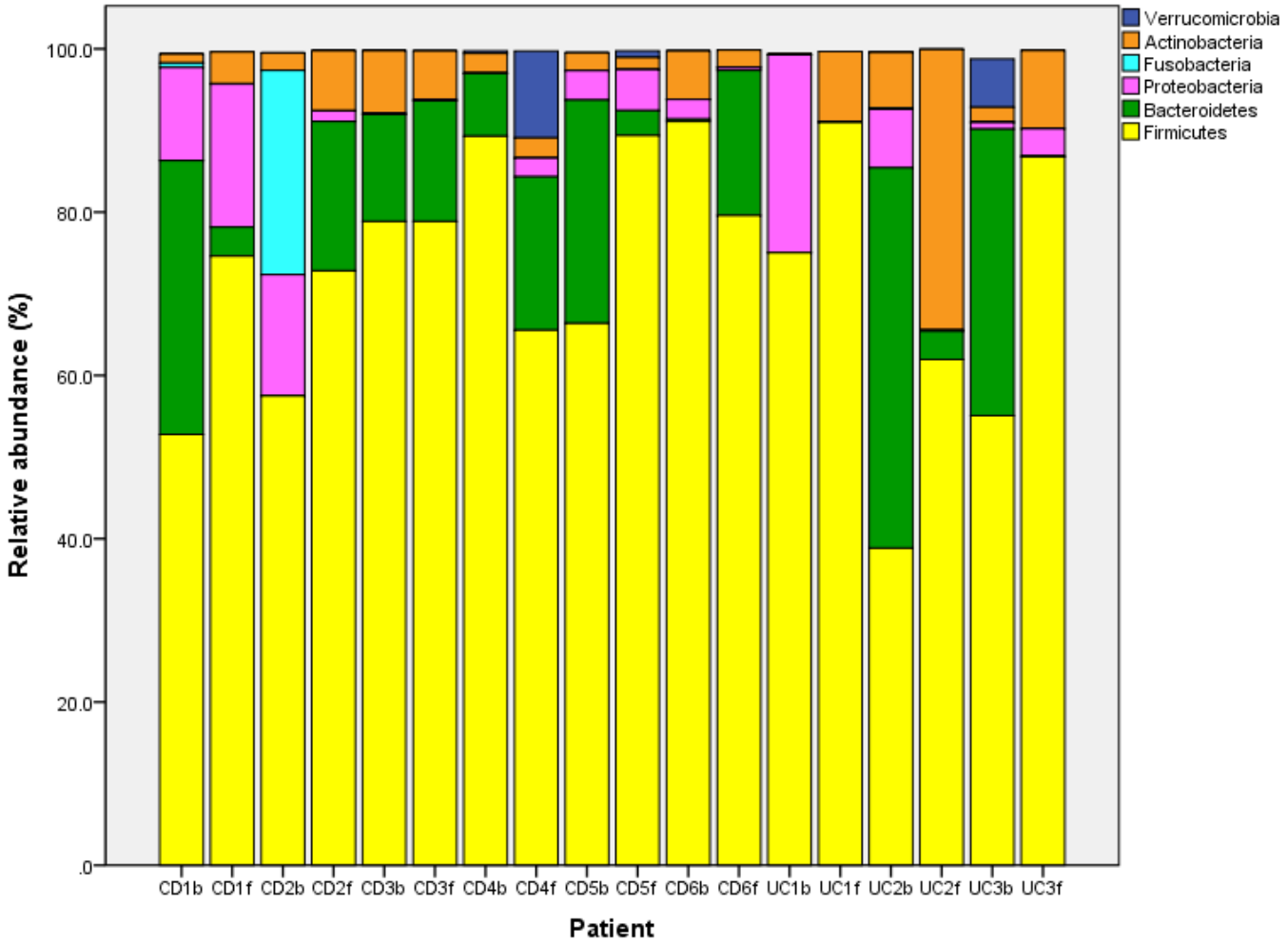

Figure 7.5 Relative abundance of bacterial phyla in paired samples. Phyla legend found on right. Data is expressed as percent (%) composition.

Table 7.7 Correlation between microbiota and biomarkers (n=24)

	Firmicutes	**Bacteroidetes**	**Proteobacteria**	**Actinobacteria**	**AA**	**PA**	**BA**
Lf	-0.224	-0.203	**.781****	-0.041	-0.174	**-.427***	-0.223
	(p=0.293)	(p=0.341)	**(p>0.01)**	(p=0.849)	(p=0.417)	**(p>0.05)**	(p=0.295)
FC	-0.09	-0.355	**.806****	-0.177	-0.16	-0.32	-0.258
	(p=0.677)	(p=0.088)	**(p>0.01)**	(p=0.408)	(p=0.454)	(p=0.127)	(p=0.224)
hsCRP	-0.154	-0.036	**.483***	-0.182	0.04	0.001	-0.156
	(p=0.471)	(p=0.869)	**(p>0.05)**	(p=0.394)	(p=0.852)	(p=0.994)	(p=0.466)

Results are expressed in correlation coefficient (p-value). Results are expressed in correlation coefficient (p-value). **hsCRP,** high sensitivity C-reactive protein, **FC,** calprotectin, **Lf**, lactoferrin **AA**, acetic acid, **PA,** propionic acid, **IBA,** isobutyric acid, **BA,** butyric acid ***Correlation is significant at the 0.05 level (2-tailed) ** Correlation is significant at the 0.01 level (2-tailed) using Pearson Correlation.**

7.5 Discussion

The primary objective of this study was to observe the fecal concentrations of SCFA and microbiota in newly diagnosed IBD patients. Within 12 months of diagnosis with IBD, fecal samples were analyzed for SCFA and microbiota to assess the difference between disease type and severity and how they related to surrogate markers of inflammation (i.e. biomarkers).

The biomarkers in this study, FC, Lf, and hsCRP, acting as surrogate markers of inflammation, fell outside the ranges of healthy levels (**Table 7.2**). Biomarker concentrations outside of the normal ranges are expected in an inflammatory condition like IBD (Vermeire, 2006).

There is limited knowledge on the abundance of fecal microbiota in newly diagnosed IBD patients. Dysbiosis, an imbalance in the composition of gut bacteria, has been observed in many ailments concerning the gut, such as IBD patients when compared with controls (Froyland, 2010; Swidsinski et al., 2002), and colorectal cancer patients, that is characterized by a significant reduction in butyrate producing bacteria, such as *F. prausnitzii* (Wu et al., 2013). These type of bacteria are of particular interest as they metabolize fibre and starch to SCFA, that helps to strengthen and maintain mucosal barrier function, along with provision of energy to the mucosal cells (Scheppach & Weiler, 2004).

Initially, levels of *Bacteroidetes* had been shown to be increased in CD patients compared with controls (Keighley et al., 1978; Ruseler-van Embden & Both-Patoir, 1983). More recently, this *Bacteroidetes have* been observed to be decreased in IBD (Frank et al., 2007). Our study showed decreased relative abundance of *Bacteroidetes*, particularly compared with fecal levels from healthy controls in other studies (Wu et al., 2013). Although not statistically significant, *Bacteroidetes* was observed to be lower in CD patients compared with UC, and lower at follow-up compared with baseline (**Table 7.4**). Additionally, relative abundance of *Bacteroidetes* have been significantly correlated with concentration of the fecal SCFA propionate (Salonen et al., 2014). Also of note, while the sample size was small (n=3), presence of *Bacteroidetes* in follow-up UC patients was almost nil (**Figure 7.2**).

A reduced abundance of the phyla *Firmicutes* has been well documented in IBD patients (Frank et al., 2007; Peterson, Frank, Pace, & Gordon, 2008; Sokol et al., 2006). Conversely, our

study showed higher levels of *Firmicutes* particularly compared with fecal levels from healthy controls in other studies (74.0 vs 38.9%) (**Table 7.4)** (Wu et al., 2013). The values were closer to those observed in tissue biopsy samples (Walker et al., 2011). It is important to note that our results present relative abundance of the microbiota, not absolute values, thus the entire population of *Firmicutes* may be decreased in this study. Although not statistically significant, relative abundance of *Firmicutes* and *Bacteroidetes* was reduced in active vs. inactive disease, while *Actinobacteria*, *Proteobacteria*, and *Fusobacteria* was increased (**Figure 7.3**). These trends warrant further attention in larger studies with greater statistical power.

F. prausnitzii has consistently been shown to be decreased in IBD and is a marker of Crohn's disease (Sokol et al., 2008, 2009). A secondary aim of this study was to observe the relationship between this important strain of gut bacteria with SCFA and fecal biomarkers. The results displayed the pivotal role *F. prausnitzii* has on gut health and inflammation. Statistically significant differences were observed between the lowest and highest quartiles of *F. prausnitzii* and observed species and butyric acid (BA) (**Table 6.5**). This means that when *F. prausnitzii* is lowest, there is less total diversity of microbiota, which was previously noted to be damaging to gut health (Sokol et al., 2008). Additionally, the strong relationship between butyric acid and *F. prausnitzii* shows that not only is production of butyrate compromised in newly diagnosed IBD, but is related to this strain of bacteria. Interestingly, the biomarkers showed a strong increasing trend across the quartiles of *F. prausnitzii*, with FC nearing statistical significance (p-value = 0.066).

Another aim in this study was the measurement of fecal short chain fatty acids (SCFA). The products of microbial fermentation of fibre, SCFA maintain health and membrane integrity in the gut, as discussed previously. The study of these molecules is complex – in fecal samples, SCFA concentration is the balance between production and absorption, that is affected by where fermentation took place, intestinal transit rate and moisture content (Salonen et al., 2014). Therefore, interpreting the results of SCFA appearance in the fecal matrix as an indication of gut environment is useful, but limited. Nonetheless, intestinal contents and epithelial tissue, that may give a better indication of SCFA abundance and expression, are not always available. Recently, Huda-Faujan et al. (2010) investigated the concentration of fecal SCFA in IBD patients. In the IBD patients the level of acetic acid, 162.0 µmol/g wet feces, butyric acid, 86.9 µmol/g wet

feces, and propionic acid, 65.6 µmol/g wet feces, were significantly lower than compared with healthy individuals, 209.7, 176.0, and 93.3 µmol/g wet feces, respectively (Huda-Faujan et al., 2010). Major findings of the Huda-Faujan et al. study agree with ours, SCFA are reduced in IBD patients (**Table 7.6**). It should be noted that the study was conducted in Malaysia where the diet consists mainly of starch. The study was also limited by a small sample size of IBD patients (n=8). None of the patients showed any diarrheal symptoms two-weeks before samples were taken, a strength in this study as moisture content could be controlled, or at least assumed to be similar.

Fecal concentrations of SCFA have been observed to be positively correlated with clinical measures of disease activity using the Truelove-Witt's criteria and CDAI (Mortensen & Clausen, 1996; Roediger et al., 1982; Treem et al., 1994). Although not statistically significant, the concentration of all major SCFA in this study were also shown to be lower in active vs. inactive IBD patients (**Figure 7.4**).

Future studies will focus on increasing the number of samples at the follow-up period to assess the resilience of bacterium through the disease course, particularly as new medications are introduced. Additionally, future studies should analyze fecal levels of SCFA to assess efficacy of nutritional interventions aimed at increasing these products (Garcia et al., 2008). Furthermore, since some prebiotics, such as inulin have been shown to promote growth of *F. prausnitzii,* dietary interventions should monitor the abundance of these bacteria (Froyland, 2010). As noted by Frank et al. (2007), a distinct subpopulation of IBD patients contain microbiota that differ entirely from study controls and IBD patients. It would be prudent for future investigators to attempt to stratify into these categories to increase quality of results.

7.6 Conclusion

Our study highlighted variations in fecal SCFA and microbiota in IBD patients, making the newly diagnosed period an important time of study. Our study displays the important relationship between microbiota and SCFA concentration - decreasing the relative amounts of *Bacteroidetes* and *Firmicutes,* responsible for producing SCFA, while increasing other bacteria, reduces the absolute amounts of SCFA, which may play a role in the pathogenesis of IBD.

CHAPTER 8
COMMENTARY

8.1 General Discussion

Biomarkers hold great promise in the management of IBD. Clinicians are presented with different challenges when treating IBD, some due the subjective nature of patient symptoms, others as a result of treating a disease that is difficult to observe. Endoscopy is invasive and expensive, while patient reported outcomes are subjective and non-specific to IBD; thus, a need for cost-effective and reliable markers of disease activity exists. The use of non-invasive, surrogate markers of disease could lessen some of these challenges, particularly in diagnosis, estimation of disease activity and inflammatory burden, and response to therapy. This thesis investigated the role of biomarkers in different domains of IBD: calculating the predictive ability within long-standing scoring measures of disease activity, evaluating the utility in a newly diagnosed cohort, and setting reference values in pregnancy. Little is known about the relationship between non-invasive markers of inflammation and nutrition in IBD; thus, studying biomarkers and nutrition was unique to our studies and an additional aim. Each chapter in this thesis builds the case for the **clinical and predictive ability of biomarkers in IBD.**

In **Chapter 4,** we examined the importance of biomarkers in a previous dataset observing the association between the SES-CD and measures of disease activity in CD. The goal was to determine the relationship between individual components of the CDAI (using both PRO- and biomarkers variables) and other disease activity variables and the SES-CD, in the effort to create a sensitive and specific model (including a PRO-exclusive model) to predict endoscopic disease activity. Our new model included biomarkers and some PRO's, which outperformed the CDAI. However, the new model has limitations. Including biomarkers requires the collection and testing of fecal and serum samples. Thus the avoidance of diagnostic testing is not averted as it would be with a PRO. However, the inclusion of biomarkers also provides an accurate surrogate measure of disease activity, which is less costly and invasive than colonoscopy. This type of model has far reaching implications; an accurate scoring index would limit the need for colonoscopy in clinical practice, reducing the burden on the health care system when monitoring IBD.

Furthermore, this model provides less expensive and more accessible primary outcomes for clinical trials.

In **Chapter 5** we studied the role of biomarkers in a longitudinal, inception cohort of newly diagnosed IBD patients. The major strength of this study was the patient population. Studying only newly diagnosed patients limits the heterogeneity (duration of disease, surgery, hospitalizations etc.) found in most IBD patient cohorts. Dietary intake was assessed through a validated FFQ. The study was limited by a small sample size, but we made some interesting observations. FC performed well in predicting endoscopic disease in both CD and UC at baseline. At diagnosis, IBD patients have a diet that fits within the AMDR for macronutrients, and is adequate in protein intake, but lacks in fibre intake. The major food groups (grain, fruit, meat, etc.) were shown to be lower in this patient population compared with reference intakes. Interestingly, glycemic index had a strong, positive correlation with fecal biomarkers. This correlation should be interpreted as hypothesis generating (i.e. diet and post-prandial glycemic response increase inflammatory biomarkers in IBD). Given the interest in diet and the post-prandial glycemic response in chronic disease future studies, using more accurate assessment of GI and GL (i.e. weighed food records, 24-hr recall with dietitian), should be completed. Additionally, both FC and hsCRP were useful in predicting disease activity in our longitudinal repeated measures analysis. As previously mentioned, the purpose for studying new diagnosis in IBD was to limit confounding in the sample population. Although our patients were newly diagnosed, the group remained heterogeneous. Differences in disease type (CD vs. UC), activity, and visit participation made the results difficult to interpret. Future studies would benefit from recruiting newly diagnosed patients with well-defined exclusion criteria (i.e. only "active" patients) at larger recruitment centers. Another limitation is concerned with classification of "newly diagnosed" patients – since patients were diagnosed within the last 12 months and placed on medical therapy, classifying them as "recently" diagnosed might be more appropriate. Future studies, particularly within the study biomarkers and microbiota, might benefit from the study of patients before the introduction of medical therapy.

In **Chapter 6**, we evaluated FC in healthy pregnancy and IBD patients. Since IBD has a peak incidence between 18 – 35 years of age, management during pregnancy is very common. Endoscopy is the gold standard is assessing disease activity, although it can be unpleasant and

painful for patients, plus time-consuming and expensive to perform. Endoscopy can be safely performed in pregnancy, but patients and clinicians still prefer to avoid invasive procedures during this time if possible, making assessment through non-invasive surrogate markers desirable. We found that FC values were normal in the HP group and significantly lower compared with the IBD group. This addition to the literature provides a much needed reference value for FC in healthy and IBD pregnancy. Clinicians can test for FC with confidence that it isn't affected by pregnancy, unlike other biomarkers of inflammation (CRP, ESR etc.).

Lastly, in **Chapter 7,** we studied the concentration of fecal microbiota and SCFA in newly diagnosed IBD patients. Although the sample size was small (n=15), we observed some meaningful descriptive trends in the inception period, such as the difference in fecal microbiota abundance between IBD and healthy patients. There is a clear trend for dysbosis in newly diagnosed patients. These observations will allow us to plan further microbiota studies, through targeting specific strains with probiotics or incorporating biomarkers as surrogate markers of inflammation.

Biomarkers hold promise in the study of nutrition and diet in IBD. Diet has been widely studied and implicated as an environmental factor in IBD, yet how it influences the disease is relatively unknown. Nutritional deficiency is prevalent in IBD, but the role nutrition plays in management and intervention is less known. This isn't due to a lack of focus – outside of pharmacologic therapeutics, nutrition is the most studied type of therapy in IBD. Historically, the bulk of nutritional studies in IBD have relied on case-control studies gathering nutritional parameters between patients and controls. As previously discussed at length in this thesis, disease activity can be assessed in IBD using clinical disease activity indices, endoscopic indices, serum serological markers, fecal markers and miscellaneous tests (Desai et al., 2007). The use of clinical scoring is partially subjective and only give an indirect measure of disease activity, while endoscopic scoring is accurate; however, it is expensive and invasive (Desai et al., 2007). Well-designed studies employing objective measures of disease, such as biomarkers, in patient populations with similar disease course could increase the level of evidence in this area. In doing so, researchers can discover the true relationship between diet and chronic disease, and design interventions to influence this modifiable risk factor.

Molodecky et al. (2011) provides an exceptional overview of several of the issues faced when investigating environmental determinants of disease, particularly in the context of IBD. Recall and diagnostic bias are the most applicable and influential to the study of diet in IBD. Recall bias is introduced into a study when the measurement of the environmental exposure is dependent on the patient's ability to recall information. Interestingly, Molodecky et al. (2011) also suggests the possibility that study patients may be more motivated than controls to examine environmental exposures in an effort to find a relationship. A clear relationship between diet and IBD is made even more difficult to ascertain due to a diagnostic bias. The time-line between environmental exposures, the onset of symptoms, the diagnosis of the disease and the introduction to an observational study all complicates the capture of dietary exposure (Molodecky, Panaccione, Ghosh, Barkema, & Kaplan, 2011). Furthermore, evidence showing that a patient will change their diet upon the appearance of symptoms complicates the study further (Maconi et al. 2010). Additionally, changes in dietary intake that may alleviate functional symptoms of disease may not be reflective of the ongoing inflammatory process (Issa & Saeian, 2011).

The use of fecal biomarkers as a study outcome and end-point would increase frequency of measurement, but more importantly, provide an objective quantification of the therapeutic and inflammatory response of a dietary intervention. Increasing the frequency of outcome measurements, made possible since biomarkers are less expensive, could highlight aspects of patient care that decrease inflammation or maintain remission. A dietary intervention aimed at modulating the intestinal inflammatory response could, hypothetically, be measured through fecal biomarker concentration.

Our research group comes from various academic backgrounds: nutrition, pharmacy, and medicine; with diverse interests, such as diet and chronic disease, medicinal therapy in IBD, and disease management in special populations like pregnancy. This group was united by a common interest – investigating existing problems with a new lens, or more specifically, a new marker. Interdisciplinary work with biomarkers brought us together as a team and allowed us to remodel an existing scoring system, study the disease in special populations like new diagnosis and pregnancy, and study environmental factors long implicated in IBD.

8.2 Conclusion

The advent of biomarkers in IBD holds promise for the management of this chronic intestinal disease. The four studies included in this thesis were a testament to the utility of biomarkers in IBD. Biomarkers were observed to be predictive of disease activity and related to important nutritional factors, like glycemic index (**Chapter 4**). They were used retrospectively to improve long-standing clinical disease activity scores in prediction of disease (**Chapter 5**). Biomarkers were shown to be significantly different in healthy and IBD pregnancy, adding them to the short list of markers useful in this special population (**Chapter 6**). Lastly, biomarkers were shown to have a strong relationship with fecal microbiota and short chain fatty acids, creating end-points for nutritional interventions aimed at influencing these communities and intestinal by-products (**Chapter 7**). Overall, the importance of biomarkers in IBD was observed throughout our studies and should be further investigated for their role in the global management of chronic disease.

8.3 Future directions

Future work should continue to focus on the study of biomarkers in newly diagnosed populations, as this timeframe holds the most promise for elucidating the cause of IBD. However, studies should only be planned in larger centers or between multiple centers, to ensure proper participant recruitment and feasibility.

In regards to creating new scores for disease activity, future studies should continue to incorporate fecal biomarkers. Additionally, model adaptation to account for variability in SES-CD subcomponents related to inter-subject variability will be explored in the future

When studying fecal microbiota, future studies will focus on increasing recruitment in pre- and post-measurements to assess the resilience of bacteria through disease course. Dietary interventions using prebiotics, such as inulin, should observe the impact on *F. prausnitzii,* a strain of bacteria involved in the production of butyrate.

In clinical and applied settings, future work will focus on incorporating biomarkers into medical triage, to take advantage of the potential fecal biomarkers have in point-of-care medical service and decision making.

When managing IBD in pregnancy, FC can be used confidently as a surrogate measure of inflammation. The relationship between this biomarker and pregnancy outcomes, such as pre-term delivery and birth weight, should be studied.

Lastly, biomarkers should be used as endpoints in nutritional interventions. They are relatively inexpensive to measure and can be detected reliably and frequently. The influence of dietary interventions on the intestinal inflammatory response could be measured through fecal biomarker concentration.

CHAPTER 9

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CHAPTER 10

APPENDICES

APPENDIX C: Ethics

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Jennifer Jones	Medicine (Gastroenterology)	09-26

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT
Royal University Hospital
103 Hospital Drive
Saskatoon SK S7N 0W8

SUB-INVESTIGATOR(S)
Anil Bedi, Lawrence J. Worobetz

SPONSORING AGENCIES
ROYAL UNIVERSITY HOSPITAL FOUNDATION - PENDING

TITLE
Endoscopic and Biomarker Predictors of Disease Course and Response to Medical Therapy in Inflammatory Bowel Disease: Establishment of a Prospective, IBD Inception Cohort

ORIGINAL REVIEW DATE	APPROVED ON	APPROVAL OF	EXPIRY DATE
23-Feb-2009	23-Apr-2009	Research Participant Information and Consent Form v.2 (23-April-2009) Tissue Banking and Genetic Testing Consent Form v.2 (23-April-2009) Appendix 10: Inflammatory Bowel Disease Questionnaire Appendix 11: Functional Assessment of Chronic Illness Therapy (FACIT) Appendix 12: SF-36 Questionnaire Appendix 13: EuroQol. 5 (EQ-5D) Appendix 14: Food Questionnaire Acknowledge Receipt of: Appendix 3: Schedule of Study Procedures - Subjects with Inactive (Stable Therapy) Crohn's Disease or Ulcerative Colitis Appendix 4: Schedule of Study Procedures - Subjects with Active (Change of Therapy) Crohn's Disease or Ulcerative Colitis Appendix 5: Simplified Endoscopic Activity Score for Crohn's Disease (SES-CD) Appendix 6: Modified Baron's Score (Endoscopic grading of Severity in Ulcerative Colitis) Appendix 7: Harvey-Bradshaw Index for Crohn's Disease Appendix 8: Simple Clinical Colitis Activity Index for Ulcerative Colitis Appendix 9: Questionnaire Description Appendix 15: Laboratory Procedures Appendix 16: Tissue Collection Consideration for Tissue Banking and Future Research Appendix 17: Future Testing	22-Feb-2010

Please send all correspondence to:

Research Ethics Office
University of Saskatchewan
Box 5000 RPO University
1607 110 Gymnasium Place
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UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval
Study Amendment

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Jennifer Jones	Medicine (Gastroenterology)	09-26

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT
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STUDENT RESEARCHER(S)
Marc Morris, Elham Rezai

FUNDER(S)
ROYAL UNIVERSITY HOSPITAL FOUNDATION

TITLE
Endoscopic and Biomarker Predictors of Disease Course and Response to Medical Therapy in Inflammatory Bowel Disease: Establishment of a Prospective, IBD Inception Cohort

APPROVAL OF	APPROVED ON	CURRENT EXPIRY DATE
Protocol revised to include healthy controls SOP for inclusion of health controls Healthy Control Participant Information and Consent Form v.1 (22-March-2013)	22-Mar-2013	12-Apr-2013

Delegated Review ☒ Full Board Meeting ☐

CERTIFICATION
The study is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL
The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face meeting. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://www.usask.ca/research/ethics_review/.

REB ATTESTATION
In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. This approval and the views of this REB have been documented in writing.

Gordon McKay, PhD., Chair
University of Saskatchewan
Biomedical Research Ethics Board

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UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval

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FUNDER(S)
UNIVERSITY OF SASKATCHEWAN - COLLEGE OF MEDICINE MEDICAL RESEARCH FUND

TITLE
Fecal Calprotectin in Pregnancy: Determination of Reference Values in Healthy Pregnancy

ORIGINAL REVIEW DATE	APPROVED ON	APPROVAL OF	EXPIRY DATE
11-Jul-2013	22-Aug-2013	Research project as outlined in the revised application for biomedical research Revised Participant Information and Consent Form v.2 (19-Aug-2013) Master List	21-Aug-2014

Delegated Review: ☒ Full Board Meeting: ☐

CERTIFICATION
The study is acceptable on scientific and ethical grounds. The Bio-REB considered the requirements of section 29 under the Health Information Protection Act (HIPA) and is satisfied that this study meets the privacy considerations outlined therein. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL
The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face) meeting. If a protocol has been reviewed at a full board meeting, a subsequent study of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://www.usask.ca/research/ethics_review/.

REB ATTESTATION
In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board has been

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APPENDIX B –Table B.1 Demographics and clinical characteristics of the newly diagnosed cohort CD patients (n=26)

Patient	Age	Gender	Location	BMI	Smoke	Therapy	CDAI	SES-CD	FC	hsCRP	Lf	SF36	IBDQ
CD1	44	M	colonic	21.6	yes	5-asa	132	12	50	4	2	105	185
CD2	25	M	ileal	26.3	no	immuno	139	7	228	23	124	107	184
CD3	22	F	ileal	20.1	no	immuno	61	9	890	13	44	100	205
CD4	19	F	colonic	19.0	no	immuno	249	21	400	32	146	110	128
CD5	52	F	ileocolonic	35.3	no	immuno	132	n.d.	1303	63	1134	n.d	0
CD6	32	M	ileal	18.0	no	anti-tnf	193	7	50	1	3	n.d	0
CD7	33	M	ileocolonic	24.8	no	anti-tnf	140	18	1487	1	115	n.d	0
CD8	52	M	ileal	27.6	no	anti-tnf	161	n.d.	612	19	281	110	201
CD9	48	M	ileocolonic	27.2	no	immuno	138	7	762	14	388	106	137
CD10	48	F	ileal	33.2	no	immuno	54	n.d.	50	1	1	101	190
CD11	47	F	ileocolonic	19.5	no	5-asa	17	n.d.	847	4	349	n.d	0
CD12	31	M	ileal	21.2	no	immuno	96	8	1348	31	452	106	183
CD13	36	M	colonic	33.7	no	none	219	5	50	3	298	96	166
CD14	35	M	ileocolonic	35.2	no	immuno	256	15	6560	10	949	100	82
CD15	28	M	ileal	33.8	no	none	-8	2	50	3	6	99	158
CD16	52	M	ileocolonic	33.9	no	immuno	59	25	1744	60	296	96	158
CD17	24	M	ileocolonic	30.4	no	immuno	354	25	7020	35	466	102	114
CD18	20	F	ileocolonic	21.7	no	anti-tnf	132	26	1362	1	216	108	209
CD19	25	F	ileocolonic	25.3	no	immuno	21	n.d.	470	4	273	106	194
CD20	22	F	ileocolonic	21.6	no	anti-tnf	109	n.d.	1236	13	360	96	175
CD21	34	F	ileocolonic	22.1	no	immuno	154	19	657	2	117	107	189
CD22	60	F	colonic	26.6	no	none	41	n.d.	2340	8	4	99	175
CD23	28	F	colonic	19.5	yes	immuno	288	14	1781	18	1	105	88
CD24	32	F	colonic	21.8	yes	immuno	351	n.d.	1781	53	1	101	87
CD25	52	F	ileal	30.3	no	immuno	85	n.d.	559	1	6	104	158
CD26	32	F	ileocolonic	29.5	n.d.	5-asa	61	8	600	31	6	105	173

Baseline demographics in 26 newly diagnosed CD patients. Disease location is confirmed by colonoscopy. **5-asa,** amino-salicylic acid, **Anti-TNF,** anti-tumour necrosis factor agent**, immuno,** immunosuppressant **CD**, Crohn's disease, **CDAI,** Crohn's disease Activity Index, **FC,** fecal calprotectin, **hsCRP**, high sensitivity C-reactive protein, **Lf,** lactoferrin **SF36,** short form 36 health survey, **IBDQ,** inflammatory bowel disease questionnaire, **n.d.**, no data.

Table B.2 Demographics and clinical characteristics of the newly diagnosed cohort UC patients (n=16)

Patient	Age	Gender	Location	BMI	Smoke	Therapy	PT	pMAYO	MAYO	FC	hsCRP	Lf	SF36	IBDQ
UC1	41	F	pancolonic	28.2	yes	immuno	9	8	10	1004	16	408	103	101
UC2	35	M	pancolonic	36.9	no	anti-tnf	10	8	11	526	62	54	88	77
UC3	49	M	left-sided	22.6	no	5-asa	3	5	6	50	2	1	83	184
UC4	70	M	left-sided	28.1	no	5-asa	7	6	7	1139	90	61	93	114
UC5	38	F	pancolonic	22.1	no	5-asa	5	3	4	323	1	117	97	147
UC6	36	M	left-sided	30.1	yes	anti-tnf	4	2	2	7660	2	878	n.d.	0
UC7	43	F	pancolonic	41.9	no	5-asa	8	9	9	1667	4	264	108	0
UC8	45	M	pancolonic	33.8	no	5-asa	0	0	0	50	0	1	108	205
UC9	54	M	pancolonic	27.2	n.d.	5-asa	2	3	3	994	3	91	112	179
UC10	50	F	left-sided	25.4	no	5-asa	13	5	8	211	1	1	n.d.	0
UC11	35	M	pancolonic	37.7	yes	anti-tnf	4	0	0	2035	5	0	n.d.	0
UC12	20	M	pancolonic	25.5	no	5-asa	10	8	11	9878	4	720	99	89
UC13	34	M	proctosigmoidal	18.6	n.d.	5-asa	6	5	7	352	6	53	108	144
UC14	35	F	proctosigmoidal	21.0	no	5-asa	8	4	6	460	2	10	104	149
UC15	55	F	pancolonic	29.6	no	5-asa	6	4	4	50	n.d.	2	101	151
UC16	37	M	pancolonic	24.0	no	5-asa	2	1	1	695	14	4	97	161

Baseline demographics in 16 newly diagnosed Ulcerative colitis patients. Disease location is confirmed by colonoscopy. **5-asa,** amino-salicylic acid, **Anti-TNF,** anti-tumour necrosis factor agent**, immuno,** immunosuppressant, **BMI,** body mass index, **PT**, Powell Tuck index, **pMAYO**, partial Mayo score, **FC,** fecal calprotectin, **hsCRP**, high sensitivity C-reactive protein, **Lf,** lactoferrin, **CD**, Crohn's disease, **SF36,** short form 36 health survey, **IBDQ,** inflammatory bowel disease questionnaire, **n.d.**, no data..

APPENDIX C: SCFA Analysis

Chromatographic Services

Department of Agricultural, Foods, and Nutritional Sciences

University of Alberta

1.0 **Title:** Short-Chain Fatty Acid (SCFA) Analysis

2.0 **Purpose:** This procedure outlines the analysis of VFAs in aqueous solutions. For this analysis, aqueous samples are combined with 25% phosphoric acid (4:1; v:v) prior to sampling for GC analysis. It is highly recommended that the addition of 25% phosphoric acid occur at the time of sample collection to avoid loss of VFAs during freezing, thawing, and sample preparation, however, this procedure assumes that this has not been done.

3.0 **Solutions:**

3.1 25% Phosphoric Acid (v/v) – 100 mL of 25% phosphoric acid is prepared by combing 29.4 mL of phosphoric acid (85%) with 70.6 ml water.

3.2 Internal Standard (Istd) - In a 100 mL volumetric flask add approximately 20 mL of 25% phosphoric acid, approximately 20 mL water and 300 µL of isocaproic acid (4-methyl-valeric acid MW 116.20 g/mol). Bring the volume up to 100 mLwith water and mix well. **Note:** It is necessary to record the weight of isocaproic acid to calculate its final concentration (µmol/ml).

3.3 Standard Solution - In a 100 mL volumetric flask add approximately 20 mL of 25% phosphoric acid, approximately 20 mL water and the following volumes of individual fatty acids:

Acetic acid	300 µL	MW 60.05 g/mol
Proprionic acid	200 µL	MW 74.08 g/mol
Isobutyric acid	50 µL	MW 88.11 g/mol
Butyric acid	100 µL	MW 88.11 g/mol
Isovaleric acid	50 µL	MW 102.13 g/mol
Valeric acid	50 µL	MW 102.13 g/mol
Caproic acid	50 µL	MW 116.20 g/mol

Bring the volume up to 100 mL with water and mix well. **Note: It is necessary to record the weight of each fatty acid in order to calculate their final concentrations (µmol/ml).**

4.0 **Sample Preparation**:

4.1 Centrifuge samples (rumen fluid, silage, etc.) at maximum speed for the container (4°C for 10 min) until a clear supernatant is obtained.
Note: For samples with lower moisture contents (e.g. feces), it may be necessary to add diluted 25% phosphoric acid (4:1, v:v: water:25% phosphoric acid) prior to centrifugation in order to obtain enough sample for analysis.

Following centrifugation, filter sample through a 0.45 µm PVDE filter.

4.2 In a GC vial (1.8 mL) combine 0.8 mL of sample, 0.200 mL of 25% phosphoric acid, and 0.200 mL of internal standard solution and mix well.

Note: It is strongly recommended that the phosphoric acid be combined with samples (4:1) at the time of sample collection.

4.3 Standards are prepared by combining 1 ml of standard solution and 0.200 ml of internal standard solution in a GC vial (1.8 mL). Mix well.

5.0 **GC Conditions:**

5.1 GC Column: Stabilwax-DA 30 meter, 0.53 mm ID, 0.5 um df (Restek Corp.).
5.2 Head Pressure: 7.5 psi.
5.3 Split vent flow: 20 mL/minute or adjusted as required.
5.4 Injector Temperature: 170ºC.
5.5 Column Temperature: 90ºC held for 0.1 min, increased to 170ºC at 10ºC/min and held for 2 min. Run time is 10 min.
5.6 Detector Temperature: 190ºC.

6.0 **Calculations:**

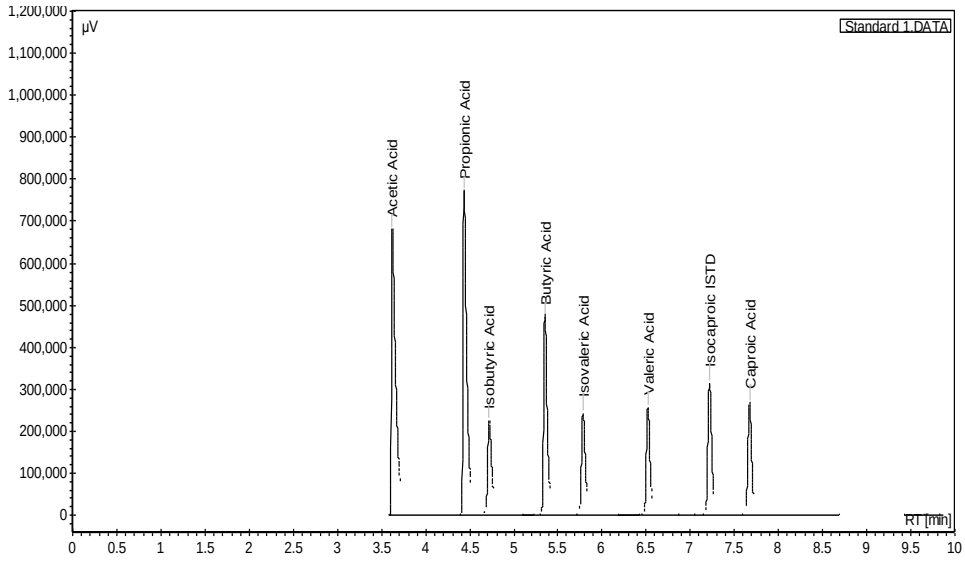
6.1 Response Factor: e.g. for Acetic Acid (A)
RRF = [A (µmol) * area Istd] ÷ [area A * Istd (µmol)]

6.2 Amount in Sample: e.g. for Acetic Acid (A)
Amt (µmol) = [area A * RRF * Istd (µmol)] ÷ area Istd

6.3 Results in 6.2 are divided by the volume of sample put into the GC vial to get the amount in µmol/ml.

7.0 **Results:**

7.1 Typical GC Chromatogram of a Standard:



7.2 Response Factors: Approximate relative response factors (RRFs) for VFAs in the standards are as follows:

Acetic acid	~4.0
Proprionic acid	2.4
Isobutyric acid	1.5
Butyric acid	1.5
Isovaleric acid	1.2
Valeric acid	1.2
Caproic acid	1.0

Note: This procedure can be extended to provide a concurrent analysis of alcohols (ethanol, butanol etc.). The standard must include these alcohols, 400 µl of ethanol and 200 µl of butanol per 100 mL. Pentanol (500 µL/100 mL) is a convenient internal standard. The initial column temperature is 35ºC and held for 3 minutes. Total run time is 15 min.

APPENDIX D: Fecal microbiota percent relative abundance in newly diagnosed IBD patients